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Identification of Nova1 as a novel modulator of microRNA function in neurons

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Abstract

The proper development, differentiation and plasticity of the nervous system require an accurate regulation at multiple levels of gene expression. One important class of post-transcriptional regulators are microRNAs (miRNAs), tiny RNA molecules that inhibit protein synthesis of target mRNAs at the level of mRNA translation and stability. MiRNAs have well documented roles in the control of neuronal development and function. Specific miRNAs, such as miR-134, regulate the local dynamic translation of mRNAs at the synapse thereby controlling activity-dependent changes in synaptic strength. MiRNAs regulate mRNA translation within a large RNA-protein complex, the microRNA-induced silencing complex (miRISC). Whereas the core components of miRISC, e.g. Argonaute (Ago) and GW182 proteins, are highly conserved, cell-type specific auxiliary proteins play important roles in the modulation of miRISC activity. The molecular mechanisms by which miRISC activity is specifically regulated in the neuronal system by such auxiliary proteins however are poorly described.

This project presents the validation and evaluation of the first large scale screening study that was performed in order to find novel RNA-binding proteins (RBPs) that regulate miRISC activity in primary neurons. The RNAi-based screen identified the RBPs Nova1, Ncoa3 and Ewsr1 as new modulators of miRISC in neurons and further confirmed the function of two previously reported miRISC interacting proteins (Ddx6, Tnrc6c). Subsequently, Nova1 was chosen for follow-up experiments directed at the elucidation of the underlying molecular mechanisms. Using luciferase reporter assays for targets of multiple neuronal miRNAs in addition to miR-134, I obtained evidence that Nova1 regulates miRNA activity in neurons in a general manner. In addition, I found that Nova1 can act as a regulator of miRNA activity irrespective of the 3'UTR context. Further data demonstrated that Nova1 interacts with Ago and the miR-134 target mRNA *Limk1*. Investigations on the functional relevance of this mechanism revealed that Nova1 is required for miR-134 mediated spine size reduction. Furthermore, I could show that Nova1 is necessary for the upregulation of *Limk1* translation upon treatment with brain-derived neurotrophic factor (BDNF), suggesting that Nova1 could be involved in the stimulus-dependent control of neuronal mRNAs. In summary, Nova1 was identified as a new modulator of miRISC activity in neurons and the underlying mechanism was

characterized in detail. The data obtained during the thesis project suggests that Nova1 is involved in the dynamic activity-dependent regulation of miRNA function in neurons.

Zusammenfassung

Die korrekte Entwicklung, Differenzierung und Plastizität des Nervensystems erfordern eine genaue Regulation auf multiplen Ebenen der Genexpression. Eine wichtige Klasse post-transkriptioneller Regulatoren sind mikroRNAs (miRNAs), winzige RNA-Moleküle, welche die Proteinsynthese von Ziel-mRNAs auf der Ebene der mRNA-Translation und -Stabilität inhibieren. MiRNAs haben wichtige Aufgaben in der Kontrolle der neuronalen Entwicklung und Funktion. Spezifische miRNAs, wie z. Bsp. miR-134, regulieren die lokale dynamische Translation von mRNAs an der Synapse, wodurch sie aktivitätsabhängige Veränderungen der Synapsenstärke kontrollieren. MiRNAs regulieren die mRNA Translation innerhalb eines großen RNA-Protein-Komplexes, des mikroRNA-induzierten Silencing Komplexes (miRISC). Während die Kernkomponenten des miRISC, wie z Bsp. Argonaute (Ago) und GW182 Proteine, hoch konserviert sind, kommen Zelltyp-spezifischen Proteinen wichtige Aufgaben in der Modulation der miRISC-Aktivität zu. Die molekularen Mechanismen, durch welche die miRISC-Aktivität speziell im neuronalen System durch solche zusätzlichen Proteine reguliert wird, sind nur unzulänglich beschrieben.

Dieses Projekt stellt die Validierung und Evaluierung der ersten umfangreichen Screening-Studie dar, die durchgeführt wurde, um neue RNA-bindende Proteine (RBP) zu identifizieren, welche die miRISC-Aktivität in primären Neuronen regulieren. Der RNAi-basierte Screen identifizierte die RBPs Nova1, Ncoa3 und Ewsr1 als neue Modulatoren von miRISC in Neuronen und bestätigte außerdem zwei zuvor beschriebene miRISC-interagierende Proteine (Ddx6, Tnrc6c). Anschließend wurde Nova1 für nachfolgende Experimente ausgewählt, welche zur Aufklärung der zugrundeliegenden Mechanismen beitrugen. Durch die Anwendung von Luciferase-Reporter Assays für Ziel-mRNAs von multiplen neuronalen miRNAs neben miR-134 erbrachte ich Nachweise, dass Nova1 die miRNA-Aktivität in Neuronen auf generelle Weise reguliert. Zusätzlich fand ich heraus, dass Nova1 als Regulator der miRNA-Aktivität in Unabhängigkeit des 3'UTR Kontextes agieren kann. Weitere Daten zeigen eine direkte Interaktion von Nova1 mit der RISC Kernkomponente Ago und eine Assoziation mit der miR-134 Ziel-mRNA Limk1. Untersuchungen über die funktionale Bedeutung dieses Mechanismus zeigten, dass Nova1 für die miR-134-vermittelte Reduzierung der Größe von dendritischen

Dornfortsätzen notwendig ist. Außerdem wurde gezeigt, dass Nova1 für die hochregulierte Translation der *Limk1* mRNA nach BDNF-Behandlung notwendig ist. Dies lässt auf eine Mitwirkung von Nova1 in der Stimulus-abhängigen Kontrolle von neuronalen mRNAs schließen. Zusammenfassend wurde Nova1 als neuer Modulator der miRISC-Aktivität in Neuronen beschrieben und die zugrundeliegenden Mechanismen genauer charakterisiert. Die während dieser Doktorarbeit erhaltenen Daten deuten zusammengenommen auf eine Beteiligung von Nova1 in der dynamischen aktivitätsabhängigen Regulation der miRNA-Funktion in Neuronen hin.

1 Introduction

1.1 Definition of microRNAs

MicroRNAs (miRNAs) are highly conserved short RNA molecules (~22 nucleotides) that constitute an important class of small non-coding RNAs. As essential regulators of post-transcriptional gene expression miRNAs are involved in a wide range of complex cellular processes including those that occur during development, differentiation and the adaptation to environmental changes. Since their first discovery in the nematode *Caenorhabditis elegans*, more than 3000 distinct miRNAs have been described in a variety of organisms ranging from simple eukaryotes to mammals. Nevertheless, the physiological role of many miRNAs is still unknown (Lee *et al.* 2001; Griffiths-Jones *et al.* 2008; Friedländer *et al.* 2014).

1.2 Mechanisms of miRNA-mediated gene silencing

MiRNAs are encoded within the genome as independent genes or in intronic regions of protein coding genes. The generation of most mammalian miRNAs is executed by the canonical miRNA pathway (Figure 1) that is described in the following chapter (reviewed by O'Carroll and Schaefer. 2013; Ha and Kim. 2014). The miRNA-mediated silencing of target mRNAs is enabled by a protein effector complex called the miRNA-induced silencing complex (miRISC) that is composed of core components and several associated factors.

1.2.1 MiRNA biogenesis pathway

MiRNA coding genes are first transcribed in the nucleus by RNA polymerase II into primary miRNA transcripts (pri-miRNAs) which may be several kilobases (kb) long (Lee *et al.* 2004). These transcripts fold into imperfectly paired, double stranded stem loop structures that are recognized in the nucleus by a specific processing complex. This so called microprocessor complex is composed of two core enzymes, the type III ribonuclease Droscha and the RNA binding protein Dgcr8 (DiGeorge syndrome critical region 8) and additional auxiliary subunits (Gregory *et al.* 2004). Dgcr8 acts as a

molecular anchor that binds to the pri-miRNA hairpin structure and recruits Drosha by a direct interaction (Han *et al.* 2006). Drosha itself cleaves the hairpin of the pri-miRNA and thereby generates a shorter molecule (~ 65 nucleotides) that is called the precursor miRNA (pre-miR). Subsequently, the pre-miR is exported to the cytoplasm by Exportin-5 in a GTP-dependent manner (Yi *et al.* 2003). In the cytoplasm, the loop region of the pre-miR is removed by the type III ribonuclease Dicer and its cofactor TRBP (trans-activation response RNA-binding protein) thereby releasing an about 22 nucleotide long double-stranded RNA (Chendrimada *et al.* 2005). The small RNA duplex is then loaded onto an Ago protein to form an effector ribonucleoprotein, known as the miRNA-induced silencing complex (miRISC). However, only one strand of the duplex (mature miRNA) serves as a guide for the recognition of the target mRNA.

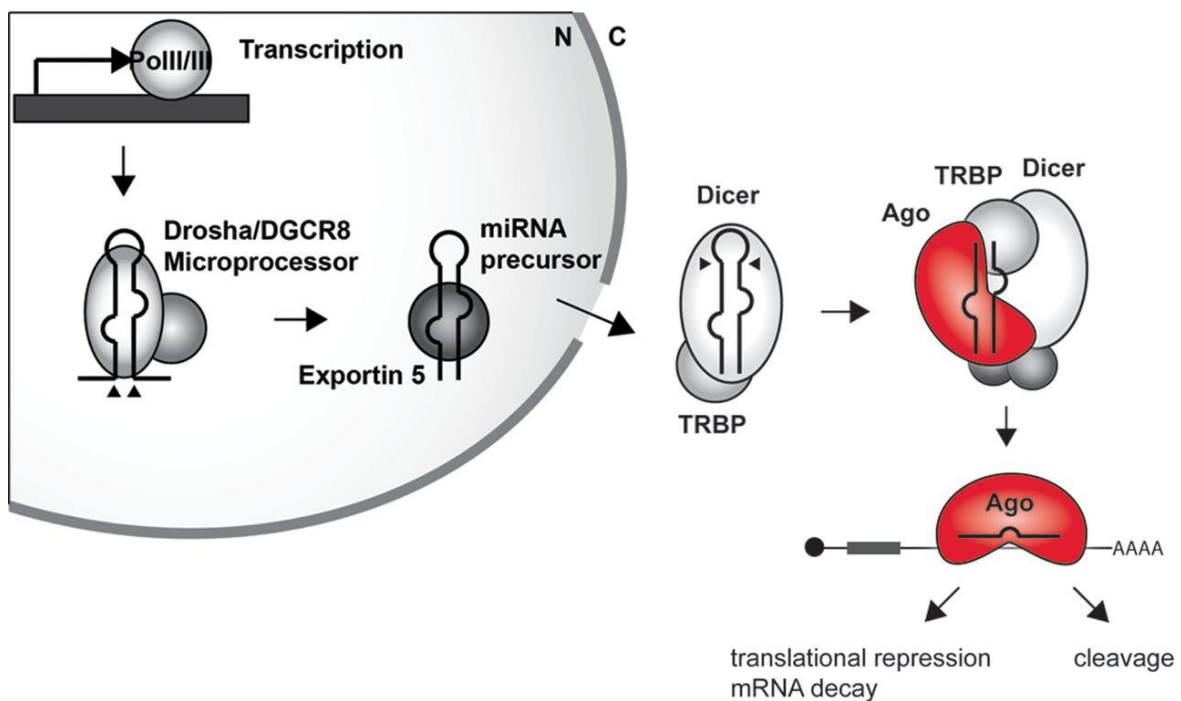


Figure 1: Overview of the canonical miRNA biogenesis pathway (Pfaff and Meister. 2013)

MiRNA genes are transcribed into primary miRNA transcripts. In the nucleus, the primary transcripts are processed by the Drosha/DGCR8 microprocessor complex to miRNA precursors. Upon export to the cytoplasm, the miRNA precursors are further processed by Dicer/TRBP to short double-stranded RNA. One strand is incorporated with Ago in the context of the miRNA-induced silencing complex (miRISC). MiRISC either directly cleaves target mRNAs or enables translational repression and decay of target mRNAs. N, nucleus; C, cytoplasm; Pol, RNA polymerase; DGCR8, DiGeorge syndrome critical region 8; TRBP, trans-activation response RNA-binding protein

The detailed mechanisms of strand selection and miRISC loading in mammals are not fully understood. Recent studies revealed that first the double-stranded miRNA is loaded onto an Ago protein that is associated with Dicer and TRBP to form the minimal RISC - loading complex (RLC) (MacRae *et al.* 2008; Noland *et al.* 2011). Upon unwinding of the duplex RNA the passenger strand (miRNA* sequence) is released and degraded. The mature miRNA is now incorporated in the mature miRISC that enables the silencing of target mRNAs.

1.2.2 MicroRNA-induced silencing complex (miRISC)

MiRNA-mediated silencing of target mRNAs is facilitated by the miRISC that is composed of two core proteins and additional factors that influence miRISC activity. The two core factors of miRISC which are indispensable for miRNA-dependent silencing are (i) one member of the Ago protein family and (ii) the direct Ago interaction partner GW182 (glycine-tryptophan protein of 182 kDa) (reviewed by Fabian and Sonenberg, 2012; Pfaff and Meister, 2013).

The mammalian genome encodes four different Ago family members (Ago1-Ago4) that are all capable of binding miRNAs. In contrast to Ago proteins from other species, human Ago proteins generally appear to lack preference for specific miRNA sequences. (Czech *et al.* 2011; Burroughs *et al.* 2011; Dueck *et al.* 2012). MiRNAs loaded in Ago guide the miRISC to specific target mRNAs in order to initiate translational repression and mRNA destabilization. Generally, miRNAs bind partially complementary to target mRNAs with mismatches at the 3' region but complementarity at the seed region (position 2 to 8) of the miRNA binding site (Bartel. 2009). In the rare case of a complete complementary base-pairing, exclusively Ago2 is able to catalyse endonucleolytic cleavage of the respective target mRNAs (Meister *et al.* 2004).

The Ago protein structure is composed of the N-terminal domain, the PAZ domain, the middle (MID) domain and the PIWI domain. The N-terminal domain is required for miRNA loading and supports the unwinding of the RNA duplex (Kwak *et al.* 2012). The PAZ domain is binding to the 3' end of the miRNA whereas the MID domain confers binding to the 5' end of the miRNA (Jinek *et al.* 2009). The PIWI domain structure is similar to that of the endonuclease RNase H and enables the cleavage of target mRNAs

at a specific nucleotide position (Song *et al.* 2004). Moreover, the PIWI domain functions as a binding platform for GW182 protein (Till *et al.* 2007).

GW182 itself serves as a binding platform for additional effector proteins which in turn induce translational repression, deadenylation and/or degradation of target mRNAs. Mammalian genomes encode three GW182 paralogues which are termed TNRC6 (trinucleotide repeat-containing protein 6) A, B and C (Eulalio *et al.* 2009). The N-terminal part of the human GW182 contains a multitude of GW repeats that enable the direct interaction with Ago. The middle part of GW182 protein is composed of a putative ubiquitin-associated (UBA) domain and a glutamine (Q)-rich domain that is responsible for localizing GW182 to processing (P)-bodies in the cytoplasm. A silencing domain within the C-terminal part that is comprised of the motifs PAM2 (poly(A)-binding protein interacting motif 2) and RRM (RNA recognition motif) facilitates interaction of GW182 with the deadenylation machinery. One of the direct interaction partners of GW182 is the poly(A)-binding protein (PABP) that is bound through the PAM2 domain. Thereby GW182 might interfere with translational initiation of target mRNAs by preventing the binding of PABP to the eukaryotic initiation factor 4G (eIF4G). Furthermore, GW182 recruits the two cytoplasmic deadenylase complexes CCR4-NOT and PAN2-PAN3, promoting deadenylation of target mRNAs (Fabian *et al.* 2012; Huntzinger *et al.* 2013). The deadenylation of mRNAs subsequently leads to the removal of the cap structure at the 5'-end by decapping that gives rise to exonucleolytic degradation by the 5'-3'-exonuclease XRN1 (Rehwinkel. 2005; Nishihara *et al.* 2013) (Figure 2).

The timing and relative importance of both translational repression and mRNA destabilization is still under debate. Recent literature points towards a direct connection between both mechanisms. It is furthermore reported that translational repression occurs first and is then followed by deadenylation and decay of mRNAs (Béthune *et al.* 2012; Meijer *et al.* 2013; Huntzinger *et al.* 2013). On the contrary, another model suggests mRNA destabilization as the dominant effect of miRNAs (Eichhorn *et al.* 2014).

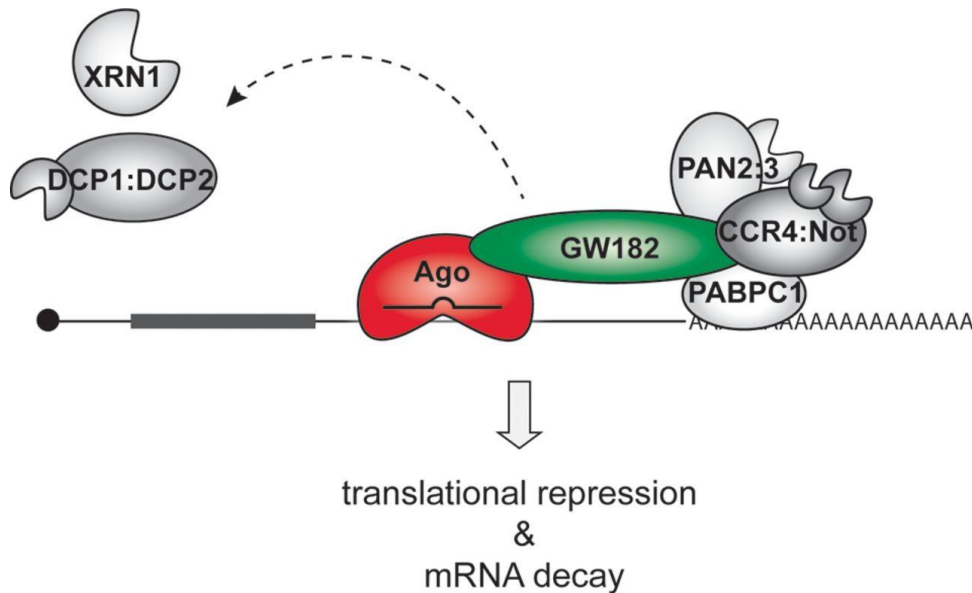


Figure 2: Mechanisms of miRNA-mediated gene silencing (Pfaff and Meister, 2013)

By binding to Ago proteins miRNAs guide the miRISC to target mRNAs. Ago recruits GW182 proteins to initiate translational repression and mRNA decay. GW182 forms a binding platform for PABPC1 and the deadenylase complexes PAN2/3 and CCR4/NOT thereby inducing deadenylation of target mRNAs. Subsequently, the mRNA is decapped by DCP1/2 enzyme activity and degraded by the 5'-3' exonuclease XRN1.

1.3 MiRNAs in the nervous system

The ability of miRNAs to control cellular processes by regulating gene expression is exceedingly important for the molecular networks that regulate the complexity of the nervous system. Numerous miRNAs are exclusively expressed or enriched in the brain and were found to be abundant in certain brain areas like the hippocampus (Bak *et al.* 2008). Neuronal miRNAs exert different roles in neurogenesis, differentiation, maturation, physiological brain function and dysfunction (reviewed by Saba *et al.* 2010; Olde Loohuis *et al.* 2012).

1.3.1 Role of miRNAs in neuronal development

Numerous studies in different model organism revealed an important contribution of miRNAs to multiple stages of neuronal development by employing loss-of-function mutants of miRNA biogenesis pathway components resulting in a global loss of mature miRNAs (reviewed by Meza-Sosa *et al.* 2014). Early studies in zebrafish demonstrated that the disruption of Dicer leads to abnormal cell morphogenesis in the brain

development. This phenotype could be rescued by introducing miR-430 suggesting an essential role of this miRNA in neuronal development (Giraldez *et al.* 2005). Another important study revealed that selective ablation of Dicer in the neocortex during mouse embryonic development results in decreased growth of the postnatal cortex and an impairment in neuronal layering (Pietri Tonelli *et al.* 2008). Furthermore, knockout of Dgcr8, caused morphological abnormalities in the central nervous system and impairments in the dendritic arborization in mice (Stark *et al.* 2008). More recent work studied the function of individual miRNAs in the developing brain. Thereby, miR-124 and miR-9 emerged as highly conserved brain-specific miRNAs that display important functions in neuronal development and differentiation of different organisms (Makeyev *et al.* 2007; Cheng *et al.* 2009; Coolen *et al.* 2013). In the developing brain miRNAs were also implicated in pathways that are required for processes involved in neuronal network formation, such as axon guidance (reviewed by Iyer *et al.* 2014).

1.3.2 Role of miRNAs in mature neurons

The main features of mature neurons are their complex structure, the connectivity between each other through synaptic contacts and the plasticity of these connections, by which neurons can adjust to changes in neuronal activity. A main research focus was on the function of miRNAs in synaptic plasticity, a cellular correlate of memory storage. Long-lasting forms of synaptic plasticity, such as long-term potentiation (LTP) or long-term depression (LTD), involve the formation/elimination of synapses and the strengthening/weakening of pre-existing synapses. Such structural modifications often happen at the level of dendritic spines, membranous protrusions from dendrites where most excitatory synapses are formed. One mechanism that is important for activity-dependent spine modifications is the local translation of specific dendritically localized mRNAs. Recent work established a critical function for dendritically localized miRNAs in the regulation of local mRNA translation and spine plasticity. Here, I will focus on three extensively studied miRNAs, miR-132, -134 and -138 (Bicker *et al.* 2014) (Figure 3).

miR-134

MiR-134 is the first miRNA that was shown to localize to dendrites. MiR-134 is required for multiple processes that involve dendrite remodeling, including the regulation of dendritic spine morphogenesis and activity-dependent dendritogenesis. In addition to

mature miR-134, pre-miR-134 was shown to localize to dendrites, suggesting a regulatory function of localized pre-miR processing. Transport of pre-miR-134 to dendrites is dependent on the RNA helicase DHX36, which interacts with the pre-miR-134 loop structure (Bicker *et al.* 2013). MiR-134 negatively regulates the size of dendritic spines by repressing the translation of *Limk1* mRNA. It was shown in hippocampal neurons that overexpression of miR-134 leads to a spine size reduction, whereas miR-134 inhibition increased spine size (Schratt *et al.* 2006). Furthermore, miR-134 regulates dendritogenesis by targeting the mRNA of the RNA-binding protein Pumilio2 (PUM2) (Fiore *et al.* 2009). Thus, both overexpression and inhibition of miR-134 block activity-dependent dendritogenesis, suggesting that regulation of miR-134 levels within a narrow window is critical for proper neural development. Recently, the mRNA of Ubiquitin-protein ligase E3A 1 (Ube3a1) was found as a target of several miRNAs encoded by the miR-379/410 cluster, including miR-134 (Valluy *et al.* 2015).

miR-132

MiR-132 belongs to the miR-212/132 family formed by four highly related miRNAs (miR-132 5p/3p, miR-212 5p/3p) which are highly conserved in the vertebrate lineage. MiR-132 promotes activity-dependent dendritogenesis and spine growth by inhibiting the expression of the Rho GTPase activating protein p250GAP, an upstream inhibitor of the Rac-PAK signaling pathway that regulates remodeling of the actin cytoskeleton (Wayman *et al.* 2008; Siegel *et al.* 2009; Impey *et al.* 2010). Dendritic miR-132 function might rely on an interaction with the RNA-binding protein FMRP, which also associates with miRISC (Edbauer *et al.* 2010). Recently, miR-132-mediated translational repression of the matrix metalloproteinase-9 (MMP-9) mRNA was shown to be involved in activity-dependent regulation of dendritic spine morphology (Jasińska *et al.* 2015). Another important target mRNA of miR-132 is methyl CpG-binding protein 2 (MeCP2) mRNA. MeCP2 is a transcriptional repressor that inhibits among other genes the expression of the neurotrophin BDNF. Since BDNF in turn activates miR-132, this constitutes a feedback control mechanism that might be involved in neuronal homeostasis (Klein *et al.* 2007; Su *et al.* 2015).

miR-138

MiR-138 is another dendritic miRNA that was identified as a regulator of spine morphogenesis. It inhibits spine growth by repressing *Lypla1* mRNA translation. The

LYPLA1 gene encodes APT1 (acyl-protein thioesterase 1), a protein with depalmitoylase enzymatic activity (Siegel *et al.* 2009). The miR-138-mediated downregulation of APT1 increases palmitoylation and therefore membrane association of the RhoA signaling activator Gα12/13. The resulting activation of RhoA modifies the actin cytoskeleton in dendritic spines which leads to spine shrinkage. Another study demonstrated that miR-138 is associated with *Lypla1* mRNA at synapses where it locally mediates its translational repression. Moreover, activity-regulated proteasomal degradation of MOV10, a component of miRISC, enables the release of *Lypla1* mRNA from miR-138-mediated translational inhibition (Banerjee *et al.* 2009). In addition, miR-138 expression was shown to correlate with short-term recognition memory in mice (Tatro *et al.* 2013) and later with memory performance in humans (Schröder *et al.* 2014). Intriguingly, mature miR-138 derives from two individual precursor forms that are encoded by two different genetic loci. The precursors pre-miR-138-1 and -2 exhibit differences in size, nucleotide sequence of the stem loop and transcription. The longer and highly expressed pre-miR-138-2 is thought to be the main source of mature miR-138 in the nervous system (Obernosterer *et al.* 2006).

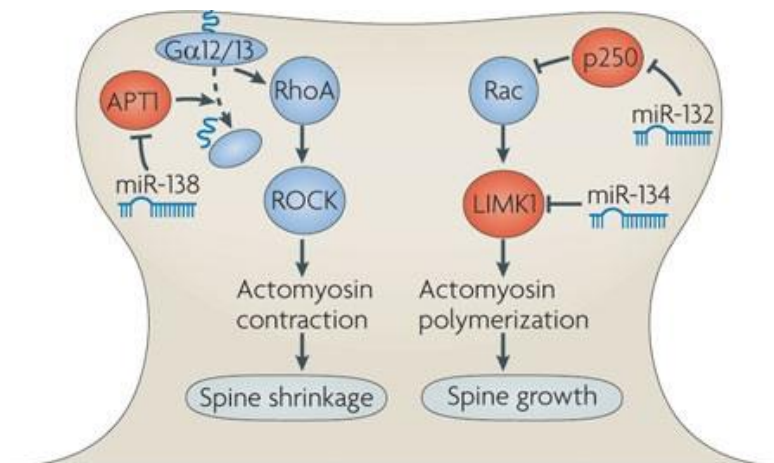


Figure 3: Control of dendritic spine size by miRNA regulatory pathways (Schratt. 2009)

Dendritic spine modifications arise from dynamic changes of the actin cytoskeleton which is controlled by two signaling pathways, the Rho/ROCK cascade and the Rac-Limk1 cascade. Several dendritic miRNAs display distinct roles in the regulation of these pathways. MiR-138 mediates the repression of the depalmitoylation enzyme acyl-protein thioesterase 1 (APT1) thereby increasing the membrane association of the RhoA signaling activator Gα12/13 leading to actomyosin contraction and subsequently reduction of spine size. MiR-134 also induces spine shrinkage by repressing the expression of Limk1 which leads to a blockage of actomyosin polymerization. In contrast, miR-132 promotes spine growth by repressing the Rac-inactivating protein p250RhoGAP that results in actomyosin polymerization.

1.3.3 Role of miRNAs in neuronal diseases

The increasing knowledge about the function of neuronal miRNAs also inspired research into possible roles of miRNAs in brain dysfunction. Cognitive impairments and learning disabilities are common characteristics of diseases of the nervous system such as Fragile X syndrome, Alzheimer disease, Parkinson's disease, Huntington disease, schizophrenia, epilepsy and autism spectrum disorders. Several studies suggest that miRNA-mediated impairments in posttranscriptional regulation are causally involved in the development of these diseases (Saba *et al.* 2010; Nowak *et al.* 2013; Alsharafi *et al.* 2015). Altered miRNA expression in post mortem brains of patients as well as in animal models of neuronal diseases further support this notion (Abu-Elneel *et al.* 2008; Moreau *et al.* 2011).

The X-chromosomally inherited neurodevelopmental disorder fragile X syndrome (FXS) was first associated with miRNAs (Jin *et al.* 2004a). Mutations in the *FMR1* gene result in the loss of the RNA-binding protein FMRP (fragile X mental retardation protein) that is involved in mRNA transport and acts as a translational repressor through association with the miRISC. Loss of FMRP leads to increased translation of mRNAs at the synapse and as a consequence might cause impairments of synaptic plasticity. It was demonstrated that *FMR1*-knockout mice display an increase in dendritic spine length and density (Nimchinsky *et al.* 2001). Moreover, a FMRP knockdown study in mouse brain implicated FMRP in the regulation of dendritic spine morphology through an association with miR-125b and miR-132 (Edbauer *et al.* 2010).

The dysregulation of individual miRNAs has also strongly been implicated in neuropsychiatric disorders such as schizophrenia and epilepsy. A high risk for developing schizophrenia-like symptoms is associated with microdeletions in the chromosomal region 22q11.2 that contains the gene encoding for the miRNA processing factor DGCR8. Deletions of this region are accompanied by alterations in brain miRNA biogenesis, thus providing a link between impaired miRNA expression and the etiology of schizophrenia (Stark *et al.* 2008). Moreover, the expression of numerous miRNAs was altered in post mortem brain samples of schizophrenia patients (reviewed by Beveridge *et al.* 2012). From these, miR-137 emerged as one of the most significant schizophrenia-associated miRNAs (Yin *et al.* 2014). In addition, multiple studies have linked alterations of miRNA expression to epilepsy (reviewed by Alsharafi *et al.* 2015). For example, expression levels of miR-132, miR-34a and miR-124 were found to be changed in experimental models of

induced epileptic seizures and in human epilepsy (Nudelman *et al.* 2010; Sano *et al.* 2012; Peng *et al.* 2013; Santarelli *et al.* 2011). Of note, miR-134 was found to be functionally relevant in the development of epilepsy because silencing of miR-134 prevents recurrent seizure occurrence in mice (Jimenez-Mateos *et al.* 2012)

1.4 Regulation of neuronal miRNAs

Since miRNAs are key regulators of activity-dependent processes in the nervous system, their turnover and function have to be precisely regulated, particularly in response to developmental or environmental cues. A particular quality of neuronal miRNAs is the activity-dependent regulation that enables cellular adaptation to changes in neuronal activity, for example during synaptic plasticity.

1.4.1 Activity-dependent regulation of neuronal miRNAs

Neuronal miRNAs are subject to distinct activity-dependent control mechanisms that operate at multiple levels, including transcription, pri-miR-processing, miRISC remodeling, miRNA turnover and function (Krol *et al.* 2010b; Treiber *et al.* 2012; Aksoy-Aksel *et al.* 2014). The various regulatory mechanisms of the miRNA pathway are either accomplished by post-translational modifications of protein core components of the miRNA pathway or RNA-binding proteins. The latter can either have enhancing or decreasing modulatory effects on miRNA function (reviewed by Loffreda *et al.* 2015). Activity-dependent regulatory processes were described for several individual miRNAs at the synapse.

For example, miR-132 was shown to be increased by different treatments that enhance neuronal activity, such as BDNF-, KCL- or bicuculline-stimulation of neurons *in vitro* (Vo *et al.* 2005; Wayman *et al.* 2008) or pilocarpine-induced seizures in rodents *in vivo* (Nudelman *et al.* 2010). The BDNF-mediated induction of the miR-212/132 locus occurs through activation of ERK1/2-signaling at the transcriptional level via two cAMP response elements (CRE) within the promoter region which are bound by the transcription factor CREB (CRE-binding protein) (Remenyi *et al.* 2010). Recently, miR-132 expression was found to be induced more physiologically in response to learning and memory formation in mice (Hansen *et al.* 2013).

The miR-134 containing cluster miR-379-410 is another example of an activity-dependent miRNA gene locus. The expression of miR-379-410 is induced upon neuronal stimulation with BDNF or KCl via activation of the transcription factor Mef2 (myocyte enhancer factor 2) that is associated with an upstream located regulatory element (Schratt *et al.* 2006; Fiore *et al.* 2009). Interestingly, the repressive activity of miR-134 within dendritic spines can be reversed by the activation of local BDNF signalling, possibly through activation of the mTOR pathway (Figure 4). This provided the first example of a local mechanism that controls miRNA function at the level of dendritic spines (Schratt *et al.* 2006). However, the underlying mechanisms are yet unknown.

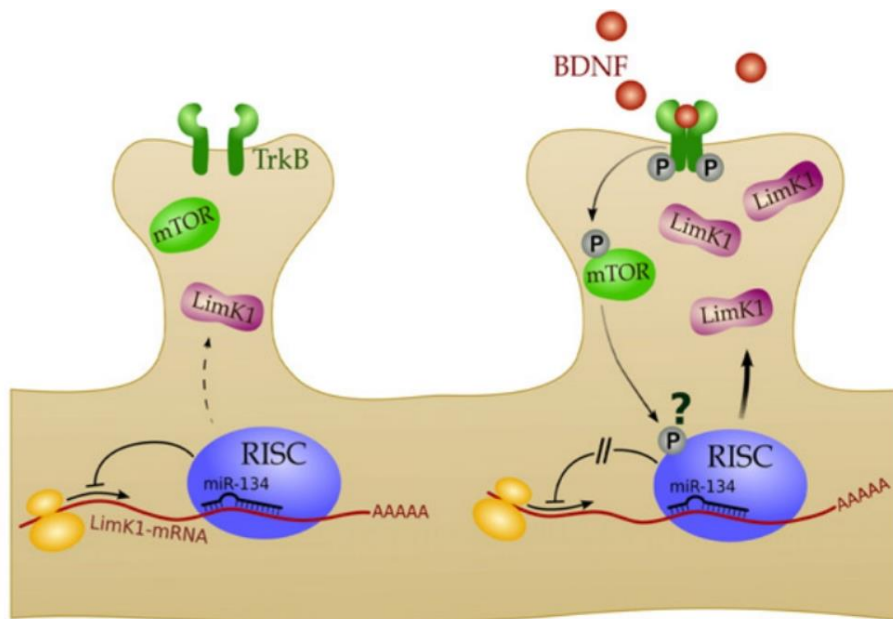


Figure 4: BDNF-induced relief of miR-134-mediated Limk1 repression (adapted from Fiore *et al.* 2008)

Within dendrites, the local Limk1 mRNA translation is repressed by active miR-134 which leads to an inhibition of spine growth. Upon extracellular stimulation with the neurotrophin BDNF, the miR-134-mediated repression is relieved and Limk1 translation is induced resulting in spine growth. BDNF release possibly induces the activation of the mTOR pathway that could lead to miRISC inactivation by protein phosphorylation.

Furthermore, there are indications that activity-dependent regulation also occurs at the level of pre-miR-processing. A recent study showed that neuronal stimulation with N-Methyl-D-aspartate (NMDA) which activates NMDA receptors changes the miRNA expression profile in cultured hippocampal neurons and also expression levels of the microprocessor components DGCR8 and Drosha (Kye *et al.* 2011). Another study linked

pre-miRNA processing to changes in neuronal activity. It was found that in response to neuronal activity inactive Dicer is partially cleaved in a calpain-dependent manner. This in turn results in an increased RNase III activity of Dicer at postsynaptic densities (Lugli *et al.* 2005). In addition, recent work demonstrated that BDNF stimulation promotes Dicer stabilization via phosphorylation of the cofactor TRBP (Huang *et al.* 2012).

A study in retina neurons revealed that miRNA levels were dynamically increased or decreased in response to light and dark conditions, respectively (Krol *et al.* 2010a). A fast turnover of miRNAs was also observed in non-retinal cultured neurons, but not in non-neuronal cells. It was further shown that inhibition of neuronal action potentials by tetrodotoxin (TTX) blocks the rapid turnover of miRNAs and that glutamate receptor stimulation induces miRNA decay. This suggests that the high turnover rate of neuronal miRNAs is determined by neuronal activity (Krol *et al.* 2010a). The molecular mechanisms underlying the regulation of activity-dependent miRNA turnover are still not known.

1.4.2 MiRNA regulation at the level of miRISC

The function of miRISC is regulated via modification of the core components, Ago and GW182, as well as via additional proteins that either positively or negatively modulate miRISC activity. Many miRISC-interacting proteins that enhance miRNA function belong to the family of DExD/H helicases, as for example Ddx6/RCK/p54 and MOV10/Armitage. RNA helicases support miRISC activity by RNA unwinding that can facilitate the incorporation of miRNAs into the miRISC as well as the interaction of miRNAs with their target mRNAs. Especially, the DEAD-box RNA helicase DDX6 is an essential miRISC component that was shown to directly interact with Ago2 thereby promoting miRNA-mediated translational repression (Chu *et al.* 2006). Moreover, Ddx6 was reported to affect miRNA-mediated silencing of target mRNAs by direct interaction with the CCR4-NOT1 deadenylase complex (Rouya *et al.* 2014).

MOV10/Armitage represents an important example of activity-dependent modification of miRISC components. The drosophila protein Armitage as well as the human homolog MOV10 (Moloney leukemia virus 10) are localized to the synapse and degraded in an activity-dependent manner by NMDA receptor stimulation. Loss of Armitage/ MOV10

stimulates the translation of synaptic mRNAs, such as *Limk1* and *Lypla1* (Ashraf *et al.* 2006; Banerjee *et al.* 2009).

Other RNA-binding proteins that facilitate miRNA-mediated repression are for example FMRP and PUF proteins. FMRP was first shown to associate with miRISC in *Drosophila* (Caudy *et al.* 2002). A later study demonstrated that FMRP cooperates with distinct miRNAs in the silencing of synaptic mRNAs in the mouse brain (Edbauer *et al.* 2010). Moreover, FMRP is involved in a mechanism that regulates mRNA translation at synapses in response to receptor activation. FMRP phosphorylation promotes the formation of an AGO2-miR-125a inhibitory complex on PSD-95 mRNA. Upon mGluR signaling the repressive activity of miR-125a can be relieved by dephosphorylation of FMRP. This leads to a release of AGO2 from the PSD-95 mRNA and a concomitant increase in PSD-95 translation (Muddashetty *et al.* 2011). The FMRP phosphorylation status also regulates the repressive activity of miR-196a, however in this case it does not affect the association between FMRP and the miR-196a target mRNA. Furthermore, FMRP directly interacts with AGO2 via a specific binding pocket within the Ago2 MID domain (Li *et al.* 2014). A recent study suggested a cooperative association between FMRP and MOV10. It was shown that FMRP directly interacts with MOV10 in an RNA-dependent manner and facilitates the association of MOV10 with RNAs in mouse brain (Kenny *et al.* 2014).

Similar to FMRP the PUF proteins (e.g. Pum1/2 in mammals) enhance the miRNA repressive function. PUF binding motifs were found to be enriched in target mRNAs around miRNA binding sites suggesting an association between PUF and the miRNA regulatory system (Galgano *et al.* 2008). The ubiquitously expressed human Pumilio homolog 1 (PUM1) protein was shown to be phosphorylated after growth factor stimulation and to subsequently modulate the secondary structure of miRNA target mRNAs. Thereby, PUM1 promotes the accessibility of miRNA seed sequences in the 3'UTR of target mRNAs (Kedde *et al.* 2010). A later study with PUF proteins from nematode and humans revealed a function in the repression of mRNA translation in cooperation with Ago. Thereby, PUF forms a complex with Ago proteins and a core translation elongation factor, eEF1A, which leads to attenuation of translational elongation of target mRNAs (Friend *et al.* 2012).

RBPs that negatively regulate miRISC function have also been reported, for example Dnd1 and HuR. Dnd1 relieves miRNA repression in human germline cells by blocking miRNA interaction with the 3'UTR of target mRNAs (Kedde *et al.* 2007; Kedde *et al.* 2010). HuR associates with the 3'UTR of mRNAs close to the miRNA binding site and promotes the dissociation of miRISC from the target mRNA (Kim *et al.* 2009; Kundu *et al.* 2012).

In addition, several other proteins (e.g. Rbm4, Smd1) were shown to be involved in the modulation of miRISC activity besides their originally described function in other cellular processes that are not related to miRNAs (Lin *et al.* 2009; Xiong *et al.* 2015).

1.5 Aim of the study

RNA-binding proteins (RBPs) that are not core components of miRISC have been shown to modify miRNA activity in non-neuronal cells (Fabian *et al.* 2012). However, miRNA modulating functions of RBPs in neurons are poorly described. This study aimed to identify new modulators of miRNA activity in neurons. Previously, our laboratory investigated the role of RBPs on miRNA activity in neurons using a large-scale RNAi-based screen in primary neuron cultures (Gabriele Siegel, PhD thesis, 2011). In my PhD thesis it was the goal to validate a subset of the positive candidates obtained from the initial screen. Next, I aimed to choose one positive candidate of the secondary screen for a detailed characterization of its potential regulatory role in miRNA function. In this regard, I intended to analyze a putative involvement in the miRNA-mediated inhibition of target mRNAs. In addition, I planned to examine the molecular mechanisms underlying the function of this candidate with respect to a potential association with miRISC. Finally, I aimed to investigate whether this RBP is involved in the activity-dependent regulation of miRNA function in neurons.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Agarose	Biozym
Ampicillin	Sigma-Aldrich
(2R)-amino-5-phosphonovaleric acid (AP5)	Tocris
Aqua-Poly/Mount	Polysciences
LB-Agar	Sigma-Aldrich
LB-Broth	Carl Roth
β -Mercaptoethanol	Sigma-Aldrich
N,N-Bis-(2-Hydroxyethyl)-2-Aminoethane Sulfonic Acid (BES)	FA Applichem
Bovine serum albumin (BSA)	New England Biolabs
Brain derived neurotrophic factor (BDNF)	Peprotech
Bromophenol blue	Sigma-Aldrich
Calcium chloride (CaCl_2)	Carl Roth
1,4-Dithiothreitol (DTT)	Sigma-Aldrich
DNA Gel Loading Dye	Thermo Scientific
Dulbecco's Phosphate-Buffered Saline (DPBS)	Life Technologies
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ethidium bromide (EtBr)	Carl Roth
5-fluorodeoxyuridine (FUDR)	Sigma-Aldrich
Glucose	Sigma-Aldrich
Hydrochloric acid (HCL)	Carl Roth
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich
Hoechst	Invitrogen
Kanamycine	Sigma-Aldrich
Laminin	BD Biosciences
LB Broth	Sigma-Aldrich
Lipofectamine 2000	Invitrogen
Magnesium acetate (MgAc)	Fisher Scientific
Magnesium chloride (MgCl_2)	Carl Roth
Methanol	VWR Chemicals

Milk powder	Carl Roth
NP-40 (Igepal)	Sigma-Aldrich
OptiPrep™ Density Gradient Medium	Sigma-Aldrich
Paraformaldehyde	Carl Roth
Poly-L-Lysine	Sigma-Aldrich
Poly-L-Ornithine	Sigma-Aldrich
Potassium acetate (KAc)	Carl Roth
Potassium chloride (KCl)	Carl Roth
Potassium hydroxide (KOH)	Carl Roth
Precision Plus Protein Dual Color Standard	Bio-Rad
Sodium chloride (NaCl)	Carl Roth
Sodium deoxycholate (NaDOC)	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	Carl Roth
Sucrose	Carl Roth
Tris(hydroxymethyl)aminomethane (Tris)	Carl Roth
Triton X-100	Carl Roth
Tween 20	Carl Roth
yeast tRNA	Sigma-Aldrich

2.1.2 Enzymes and inhibitors

Restriction enzymes

<i>AscI</i> , <i>EcoRI</i> , <i>HindIII</i>	New England Biolabs
<i>BamHI</i> , <i>BsrGI</i> , <i>DpnI</i> , <i>MssI</i> , <i>NotI</i> , <i>SacI</i> , <i>XbaI</i>	Thermo Scientific

Other enzymes

Benzonase® Nuclease	Sigma-Aldrich
DNA Polymerase I, Large (Klenow) Fragment	New England Biolabs
<i>Pfu</i> Plus! DNA Polymerase	Roboklon
RNase A	Ambion
Shrimp alkaline phosphatase	Promega
T4 DNA ligase	Invitrogen
Turbo DNase	Ambion

Inhibitors

Complete Protease inhibitors	Roche
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Phosphatase inhibitors cocktail 2 and 3	Sigma-Aldrich
SUPERase inhibitor	Ambion

2.1.3 Lab equipment

Amicon Ultra-15 centrifugal filter with Ultracel-100 membrane	Merck Millipore
Cell culture dishes (15 cm ²)	Corning
Cell culture flasks (25, 75, 175 cm ²)	Sarstedt
Cell culture plates (24-well, 6-well)	Corning
Cell-lifter	Corning
Centrifuge JC-MC	Beckman Coulter
Coverslips	Carl Roth
Curix 60 Tabletop processor	Agfa
Digital gel documentation system E-Box	Vilber
Dounce potter (15 ml)	Sartorius
Eppendorf reaction tubes (1.5 ml/2 ml)	Eppendorf
GloMax R96 Microplate Luminometer	Promega
Hyperfilm ECL	GE Healthcare
Intell Mixer RM-2L	Elmi
LSM 5 Pascal microscope	Zeiss
Magnetic Particle Concentrator DynaMag™ -2	Invitrogen
Microscope slides	neoLab
Microtiter plate (96-well)	Nunc
Mini-PROTEAN® Tetra Vertical Electrophoresis Cell	Bio-Rad
Nano Drop 2000c	Thermo Scientific
Needle (26G)	B.Braun
Protein G beads	Sigma-Aldrich
Protein G Dynabeads®	Life technologies
PVDF membrane	Millipore
Rotor type 70 Ti	Beckman Coulter
Sonifier 250	Branson
Step One Plus instrument	Applied Biosystems
Thermal cycler S1000™	Bio-Rad
Tubes type 70 Ti	Beckman Coulter
Ultracentrifuge Optima XE-90	Beckman Coulter

2.1.4 Kits

Amersham ECL Prime Western Blotting Detection Reagent	GE Healthcare
Dual-Luciferase® Reporter Assay System	Promega
iScript cDNA synthesis Kit	Bio-Rad
iTaq SYBR Green Supermix with ROX	Bio-Rad
mirVana™ Isolation Kit	Ambion
NucleoBond® Xtra Midi Kit	Macherey-Nagel
Pierce™ BCA Protein Assay Kit	Thermo Scientific
QIAquick Gel Extraction Kit	Qiagen
QIAquick Purification Kit	Qiagen

2.1.5 Buffers and solutions

2x HBS:	50 mM HEPES 10 mM KCl 12 mM Glucose 280 mM NaCl 1.5 mM Na ₂ HPO ₄ pH 7.05
2x BBS:	50 mM BES 1.5 mM Na ₂ HPO ₄ 280 mM NaCl pH 6.95
Tris/NaCl:	50 mM Tris 150 mM NaCl pH 8.0
PBS-MK:	1 x DPBS 1 mM MgCl ₂ 2.5 mM KCl
5x Annealing buffer:	100 mM KAc 30 mM HEPES-KOH, pH 7.4 2 mM MgAc
50x TAE buffer:	2 M Tris-acetate 50 mM EDTA
KCM solution:	100 mM KCL 30 mM CaCl ₂ 50 mM MgCl ₂

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HLB:	10 mM KCL 1 mM EDTA 1 mM DTT 10 mM Tris-HCl pH 7.5
10x RIPA:	0.5 M Tris pH 8.0 1.5 M NaCl 10% Triton X-100 5% NaDOC 0.5% SDS 20 mM EDTA
lysis buffer (protein extraction):	150 mM NaCl 50 mM Tris pH 7.5 1% Triton X-100
4x Laemmli sample buffer:	250 mM Tris-HCl (pH 6.8) 8% SDS 40% glycerol 8% β -mercaptoethanol 0.04% bromophenol blue
TBS-T:	50 mM Tris 150 mM NaCl 0.1% Tween 20 pH 7.5
Running buffer:	25 mM Tris 192 mM glycine 0.1% SDS
Blotting buffer:	25 mM Tris 192 mM glycine 20% methanol
lysis buffer (co-IP):	20 mM Tris pH 7.5 150 mM NaCl 1% NP-40 2 mM EDTA
lysis buffer (RNA-IP):	10 mM HEPES, pH 7.5 200 mM NaCl 30 mM EDTA 0.5% Triton X-100 0.5 U/ μ l SUPERase inhibitor
GDB:	20 mM Sodium phosphate buffer, pH 7.4 450 mM NaCl 0.3% Triton X-100 0.1% gelatine

2.1.6 Cells and culture media

Prokaryotic cells

Escherichia coli (*E. coli*) (XL1-Blue)

LB medium: LB-Broth

LB Agar plates: 1.5% LB-Agar in LB medium

Eukaryotic cells

Human embryonic kidney 293 (HEK293) cells

Growth medium:	Minimum essential medium (MEM)	Gibco
	10% fetal calf serum (FCS)	Invitrogen
	2 mM L-Glutamine	Invitrogen
	100 U/ml Penicillin/Streptomycin	Invitrogen

Primary neuronal cells prepared from embryonic day 18 Sprague Dawley rats (Charles River Laboratories)

Growth medium (NB+):	Neurobasal medium (NB)	Invitrogen
	2% B27 supplement	Invitrogen
	1 mM GlutaMax	Invitrogen
	100 U/ml Penicillin/Streptomycin	Invitrogen

2.1.7 Oligonucleotides

Oligonucleotide sequences are notated from 5' to 3' direction.

Primers used to clone 3'UTRs to pGL4:

LIN41-FW	GATCTCTAGACACTTCTCTTGCTCTTTAC
LIN41-REV	GATCGCTAGCTTTATTCGAATTATGTTATCAG
LIMK1-FW	CTTCTAGAGATACTTGGAGGATAGACCCTCACC
LIMK1-REV	GCCCCGACTCTAGCTAGCGGGAGCACAGAATTGAT

Primers used for site directed mutagenesis:

Nova1 ^R -FW	GATTTTATCCAGGTACACCGAGCGGGTTTGCTTGATCCAGG
Nova1 ^R -REV	CCTGGATCAAGCAAACCCGCTCGGTGGTACCTGGATAAAAATC

Oligonucleotides used for cloning rAAV-Nova1 shRNA:

Nova1-shRNA-fw1 GATCCCCGGTACTACTGAGAGGGTTTTCAAGAGA
 Nova1-shRNA-fw2 AAACCCTCTCAGTAGTACCTTTTTA
 Nova1-shRNA-rev1 AGCTTAAAAAGGTACTACTGAGAGGGTTTTCTCTTGAA
 Nova1-shRNA-rev2 AAACCCTCTCAGTAGTACCGGG

Primers used for cloning of NHA-fusion constructs:

NHA-Nova1-Xba1-FW TCTCTAGAATGGCGGCAGCTCCCATTCAGCAGAACG
 NHA-Nova1-Not1-REV CCGGTGGCGGCCGCGTCAACCCACTTTCTGAGGATTGGCAG
 NHA-Nova1-del1-Not1-REV CTGTGGATCCTCTGCGGCCGCCTGCTAGATAAGTTCAACAG
 NHA-Nova1-del2-Xba1-FW TGAATCTAGAATCCAGAAGATACAAGAGGATCCACAGAGTG
 NHA-EGFP-FW CCACCGGTCGACACCATGGTGAGCAAGGGCG
 NHA-EGFP-REV ATCTAGAGTCGCGGCCGCTTTACTTGTAC

Primers used for qPCR:

U6 snRNA FW CTCGCTTCGGCAGCACA
 U6 snRNA REV AACGCTTCACGAATTTGCGT
 GAPDH FW GCCTTCTCTTGTGACAAAGTGA
 GAPDH REV CCGTGGGTAGAGTCATACTGGAA
 Limk1 FW CCTCCGAGTGTTTGTCTGA
 Limk1 REV CAACACCTCCCCATGGATG
 Rgs4 FW ACAAGCCGGAACATGTTAGAG
 Rgs4 REV AGACTTGAGGAAACGACGGT

2.1.8 RNA molecules**Table 1: MiRNA sense and antisense sequences**

miRNA	Sequence (5' – 3')
miR-134 sense	Phospho-UGUGACUGGUUGACCAGAGGGA
miR-134 antisense	Phospho-CCUCUGGUCAACCAGUUAUACU
miR-138 sense	Phospho-AGCUGGUGUUGUGAAUCAGGCCG
miR-138 antisense	Phospho-GCCUGAUUCACAACACCAGAUUU

MiRNA sense and antisense oligos (Table 1) (Integrated DNA Technologies) were used for miRNA duplex annealing (chapter 2.3.1).

Table 2: SiRNA subset of custom siRNA library (Ambion)

siRNA	siRNA sense sequence	siRNA antisense sequence
Ewsr1 siRNA-1	GGAU AUGCACAG ACCACCCtt	GGGUGGUCUGUGCAUAUCCtt
Ncoa3 siRNA-2	GGAGACAGUGAGACAGAUAtt	UAUCUGUCUCACUGUCUCCtt
Nova1 siRNA-3	GGUACUACUGAGAGGGUUUtt	AAACCCUCUCAGUAGUACCtg
Nxf1 siRNA-1	CGAUUUCCCAAGUUAUUACtt	GUAUAACUUGGGAAAUCGtt
Nxt1 siRNA-2	GCCACUUUAGUAUGGAAUGtt	CAUCCAUACUAAAGUGGCtg
Rbm25 siRNA-2	GGACAUUUCCGUAGAUUUtt	AAAUCUACGGAAAAUGUCCtc
Rpl5 siRNA-3	GGAGAUGUAUAAGAAAGCUtt	AGCUUUCUUAUACAUCUCCtc
Tnrc6c siRNA-3	GGUUCAAGCACAGCUUUUGtt	CAAAAGCUGUGCUUGAACCTg
U2af1 siRNA-2	CCUUUAGCCAGACCAUUGCtt	GCAAUGGUCUGGCUAAAGGtt

2.1.9 Plasmids

Luciferase reporter plasmids

The pGL4.13 vector (Promega) encodes the luciferase reporter gene luc2 (firefly). The vector contains an SV40 early enhancer/promoter. The firefly reporter constructs were generated by PCR amplification and/or restriction digest of the corresponding 3'UTRs (Table 3) from pGL3-3'UTR reporter constructs. The 5'overhangs of the resulting inserts were filled by Klenow reaction (chapter 2.3.5). The 3'UTRs were inserted downstream of the firefly gene into the *Xba*I restriction site of pGL4.13. The 138-sponge-luc reporter was generated by inserting six binding sites for miR-138 carrying a bulge at position 9-12 (Ebert *et al.* 2007) into pGL4.13 vector. The control (ctrl) sponge was created by inserting the reverse sequence of the 138 sponge into pGL4.13.

In order to generate the renilla luciferase vector pGL4-RL, the renilla luciferase gene was excised by restriction digest from the plasmid pRL-TK and inserted into pGL4.13 by replacing the firefly coding sequence.

Table 3: Luciferase reporter plasmids

Luciferase reporter plasmid	3'UTR / Reference	Restriction sites used for cloning 3'UTR
pGL4-UBE3A	UBE3A rat (606 bp) (Valluy <i>et al.</i> 2015)	<i>Bam</i> HI, <i>Xba</i> I
pGL4-APT1	APT1 mouse (Siegel <i>et al.</i> 2009)	<i>Asc</i> I, <i>Eco</i> RI
pGL4-LIN41-WT	LIN41 <i>C. elegans</i> (D. Bartel)	<i>Not</i> I, <i>Mss</i> I
pGL4-LIN41-mutant	LIN41 <i>C. elegans</i> (D. Bartel)	<i>Not</i> I, <i>Mss</i> I
pGL4-LIMK1-WT	LIMK1 rat (Schratt <i>et al.</i> 2006)	<i>Xba</i> I, <i>Nhe</i> I
pGL4-LIMK1-mutant	LIMK1 rat (Schratt <i>et al.</i> 2006)	<i>Xba</i> I, <i>Nhe</i> I
pGL4-IQGAP1-WT	IQGAP1 human (D. Bartel)	<i>Sac</i> I, <i>Xba</i> I
pGL4-IQGAP1-mutant	IQGAP1 human (D. Bartel)	<i>Sac</i> I, <i>Xba</i> I
pGL4-HMGA2-WT	HMGA2 mouse (D. Bartel)	<i>Xba</i> I, <i>Not</i> I
pGL4-HMGA2-mutant	HMGA2 mouse (D. Bartel)	<i>Xba</i> I, <i>Not</i> I
pGL4-138-sponge	6x miR-138 binding site (S. Bicker, K. Weiß)	<i>Bsr</i> GI, <i>Hind</i> III
pGL4-ctrl-sponge	6x miR-138 binding site reversed (S. Bicker, K. Weiß)	<i>Bsr</i> GI, <i>Hind</i> III

ShRNA expression plasmids

In order to generate shRNA expression vector pAAV-Nova1 shRNA, DNA oligonucleotides (chapter 2.1.7) were annealed followed by ligation into the *Bgl*II and *Hind*III restriction sites of the pAAV vector (Christensen *et al.* 2010). The pAAV vector contains inverted terminal repeats (ITRs) that flank a U6 promoter driven shRNA sequence upstream of a chicken β -actin (CBA) promoter driven GFP gene.

Table 4: ShRNA expression plasmids

shRNA expression plasmid	Reference
pSuper basic	Oligoengine
pSuper-control shRNA	Störchel and Thümmeler <i>et al.</i> 2015
pSuper-Nova1 shRNA	Störchel and Thümmeler <i>et al.</i> 2015
pAAV-control shRNA	pAM/U6-sHRNA-EGFP-CBA-hrGFP (obtained from M. Schwarz)
pAAV-Nova1 shRNA	generated from pAAV-control shRNA

GFP-Nova1 overexpression vector

To generate the vector expressing an shRNA-resistant Nova1 protein (Nova1^R), silent mutations were introduced into the GFP-Nova1 expressing vector pEGFP-C1-Nova1 (G. Siegel) by site directed mutagenesis (chapter 2.3.8) using the primer Nova1^R-FW and Nova1^R-REV resulting in the vector pEGFP-C1-Nova1^R.

Table 5: GFP-Nova1 overexpression plasmids

GFP-Nova1 overexpression plasmid	Reference
pEGFP-C1	Clontech
pEGFP-C1-Nova1	Störchel and Thümmeler <i>et al.</i> 2015
pEGFP-C1-Nova1 ^R	generated from pEGFP-C1-Nova1

Plasmids for tethering assay

To generate pCI-neo-NHA-Nova1 and pCI-neo-NHA-GFP, the respective coding sequences were amplified by PCR from the vector pEGFP-C1-Nova1 and pEGFP-N1. Subsequently, the PCR product was inserted into the vector pCI-Neo-NHA downstream of the N-HA peptide sequence by use of the restriction sites that are indicated in the primer name (chapter 2.1.7).

Table 6: Plasmids used for tethering assays

Tethering assay plasmid	Reference
RL-5boxB	obtained from R. Pillai
pCI-neo-NHA	obtained from R. Pillai
pCI-neo-HA-Tnrc6c	obtained from R. Pillai
pCI-neo-NHA-Tnrc6c	obtained from R. Pillai
pCI-neo-NHA-Nova1	generated from pCI-Neo-NHA
pCI-neo-NHA-GFP	generated from pCI-Neo-NHA
pCI-neo-NHA-Ncoa3	Störchel and Thümmeler <i>et al.</i> 2015

2.1.10 Antibodies

Table 7: Antibodies used for Western blot (WB), immunocytochemistry (ICC), RNA-IP and co-IP

Antibody	Species	Source	Application/ Dilution
Primary antibodies			
anti-MAP2	mouse	clone HM-2, Sigma-Aldrich	ICC/ 1:2,000
anti- α -Tubulin	rabbit	cat.# 2144, Cell Signaling	WB/ 1:5,000
anti- β -Actin	mouse	clone AC-15, Sigma-Aldrich	WB/ 1:10,000
anti-HDAC2	rabbit	ab32117, Abcam	WB/ 1:5,000
anti-Limk1	mouse	clone 42, BD Biosciences	WB/ 1:200
anti-Nova1	rabbit	Merck Millipore cat.# 07-637	WB/ 1:1,500; ICC/ 1:250
anti-Nova1	rabbit	ab183723, Abcam	RNA-IP
anti-Nova-pan	rabbit	obtained from R. Darnell	WB/ 1:5,000
anti-pan-Ago	mouse	clone 2A8, Merck Millipore	co-IP/ 1:200
anti-Ago2	rat	clone 6F4, obtained from G. Meister	WB/ 1:10 (serum)
anti-HA	rabbit	ab9110, Abcam	ICC/ 1:1,000
rabbit IgG	normal	Santa Cruz Biotechnology	RNA-IP
mouse IgG	normal	Santa Cruz Biotechnology	co-IP
Secondary antibodies			
anti-mouse HRP	rabbit	Calbiochem	WB/ 1:20,000
anti-rabbit HRP	goat	Calbiochem	WB/ 1:20,000
anti-rat HRP	goat	Calbiochem	WB/ 1:20,000
Alexa 488 anti-rabbit	goat	Life technologies	ICC/ 1:1,000
Alexa 647 anti-mouse	donkey	Life technologies	ICC/ 1:1,000
Alexa 546 anti-mouse	goat	Life technologies	ICC/ 1:1,000

2.2 Cell culture methods

2.2.1 Primary neuronal cell culture

Dissociated primary cortical and hippocampal neurons were prepared from embryonic day 18 rats as described in Schratt *et al.* (2004). After dissociation cortical neurons were

directly plated on Poly-L-Ornithine (Sigma) coated 24-well or 6-well cell culture plates. Hippocampal neurons were plated on nitric acid-treated coverslips that were coated with Poly-L-Lysine and Laminin in a 24-well format. Both types of primary neurons were cultured in Neurobasal medium (NB+) at 37 °C and 5% CO₂. Primary neurons were routinely prepared by Gertraud Jarosch, Eva Becker, Renate Gondrum and Heinrich Kaiser.

For the analysis of subcellular fractions, hippocampal neurons were cultured in medium supplemented with 10 µM of 5-fluorodeoxyuridine (FUDR) to prevent the growth of glial cells. For BDNF stimulation, cortical neurons were treated with BDNF at a final concentration of 100 ng/ml for 5 min to 5 h before cell lysis.

2.2.2 Human embryonic kidney 293 cell culture

Human embryonic kidney 293 (HEK293) cells were cultivated in 6-well- or 12-well-culture plates at subconfluent density in minimum essential medium (MEM) supplemented with FCS, L-Glutamine and Penicillin/Streptomycin at 37 °C and 5% CO₂.

2.2.3 Transfection of neurons with Lipofectamine 2000

Cultured neuronal cells were transfected with the Lipofectamine 2000 transfection reagent. Per well (24-well format), a total of 1 µg of RNA or DNA was mixed with a 1:66 dilution of Lipofectamine 2000 in 100 µl Neurobasal medium (NB, without supplements). The mixture was incubated at room temperature (RT) for 20 min, then diluted 1:5 in NB medium and gently applied to the cells. After 2 h of incubation at 37 °C and 5% CO₂, the cells were washed with NB. Subsequently, the cells were incubated with the NMDA-receptor antagonist (2R)-amino-5-phosphonovaleric acid (AP5, 20 µM) in NB+ for 45 min at 37 °C. After an additional washing step with NB the cells were provided with one third of fresh NB+ medium mixed with two third of medium cells were incubated in before (conditioned medium) that was collected before the transfection procedure.

2.2.4 Transfection of HEK293 cells with calcium-phosphate

HEK293 cells were transfected at a confluency of 60% using calcium-phosphate. Per well (12-well format) a total amount of 3.2 µg of DNA was mixed with 6 µl of 2 M CaCl₂ in H₂O (total volume of 50 µl). Then 50 µl of 2x HBS were added to the mixture while

slowly vortexing. The mix was incubated for 3 min at RT and gently applied drop-wise to the cells medium. After 5 h incubation at 37 °C and 5% CO₂ the medium was replaced with fresh MEM. The cells were lysed after incubation for 18-24 h.

2.2.5 Production of recombinant adeno-associated virus (rAAV)

Recombinant adeno-associated viruses (rAAV) expressing either Nova1 shRNA or control shRNA were generated by co-transfection of the shRNA expressing pAAV plasmid and the helper plasmids pDP1 and pDP2 (Grimm et al. 2003) into HEK293 cells using calcium-phosphate. The cells were plated onto 15 cm² cell culture dishes and transfected at 80% confluency. The DNA mix was prepared for a total of 8 dishes (96 µg pAAV, 192 µg pDP1, 192 µg pDP2) and mixed with 2.5 ml 2 M CaCl₂ and 17.5 ml H₂O. After splitting the solution to 8 vessels (2.5 ml each) 2.5 ml of 2x BBS were added. The solution was gently mixed by inverting the tube and incubated at RT for 3 min. 20 ml of MEM were added and the mixture was applied to the cells after removing the complete culture medium. Following 5 h of incubation at 37 °C and 5% CO₂, the cells were washed with 25 ml fresh MEM. After 3 days, the cells were harvested with a cell-lifter and collected in 4 tubes. The pelleted cells from 8 plates were united in 25 ml DPBS and resuspended in 8.5 ml Tris/NaCl. 2 µl of Benzonase (25 U/µl) were added followed by 500 µl of 10% NaDOC. After incubation for 30 min at 37 °C 584 mg NaCl were dissolved in the cell solution followed by 30 min of incubation at 56 °C. The cells were frozen overnight at -20 °C, thawed and centrifugated for 30 min at 16,000 g. After another freeze-thaw cycle the virus was purified by discontinuous iodixanol gradient centrifugation (Zolotukhin *et al.* 1999). The sample was loaded onto the gradient (7 ml of 15%, 4 ml of 25%, 3 ml of 40% and 3 ml of 54% iodixanol) in 70 Ti rotor tubes and spun in the ultracentrifuge for 2 h at 55,000 rpm (rotor type 70 Ti). To recover the virus, the 40% phase and interphase between 40% and 54% were aspirated with a syringe and 1:1 diluted in PBS-MK. The virus was concentrated through filter (Amicon Ultra-15 centrifugal filter with Ultracel-100 membrane) by centrifugation at 2,000 g.

2.2.6 rAAV infection of neurons

In order to yield a high number of shRNA expressing cells in the neuronal culture, cells were infected with shRNA expressing recombinant adeno-associated virus (rAAV). Cortical neurons were infected at 4 days in vitro (DIV) with shRNA expressing rAAV

(2×10^6 IFU (infectious units)/ml). Virus infected cells were lysed 10 days after infection (14 DIV).

2.3 Molecular methods

2.3.1 Annealing of miRNA duplex

MiRNA duplex RNA was synthesized by annealing single stranded miRNA sense and miRNA antisense strands (Table 1). 20 μ l of each strand (100 μ M) were mixed with 10 μ l 5x Annealing buffer. The mixture was incubated in the thermal cycler for 2 min at 95 °C, for 1 h at 37 °C and cooled down to 4 °C.

2.3.2 Polymerase chain reaction (PCR)

The amplification of specific DNA fragments for cloning was performed by polymerase chain reaction (PCR) using the *Pfu*Plus! DNA Polymerase. In accordance with the manuals' instruction, PCR reactions were set up as follows and incubated in a thermal cycler with the following program.

PCR reaction (50 μ l):

5 μ l 10x Pfu buffer
4 μ l 2.5 mM dNTP mix
2 μ l 10 μ M forward primer
2 μ l 10 μ M reverse primer
1 μ l plasmid DNA (50 ng)
0.5 μ l *Pfu*Plus! DNA polymerase (2.5 U)
35.5 μ l ddH₂O

PCR program:

Initial denaturation	95 °C, 3 min	
Denaturation	95 °C, 50 sec	} 35 x
Annealing	50 - 68 °C, 30 sec	
Extension	72 °C, 1 min/kb	
Terminal extension	72 °C, 7 min	
	4 °C, ∞	

2.3.3 Agarose gel electrophoresis

The separation of DNA fragments according to their size was performed by agarose gel electrophoresis. The DNA samples were mixed with DNA Gel Loading Dye (Thermo Scientific™) and loaded onto a 1% agarose gel in 1x TAE buffer containing ethidium bromide (1:200). The gel was run at 120 V. The bands were visualized by UV-light in a digital gel documentation system.

2.3.4 Purification of plasmid DNA and PCR products

Restricted DNA fragments were separated by agarose gel electrophoresis and the respective bands were excised from the gel followed by purification using the QIAquick Gel extraction kit. PCR products were purified by use of the QIAquick purification kit according to the manuals' instructions.

2.3.5 Restriction digest, dephosphorylation, Klenow reaction and ligation of DNA

For cloning of recombinant DNA constructs, plasmid DNA or PCR-products were digested with restriction enzymes (chapter 2.1.2) according to the manuals' instructions.

In order to generate blunt ends of fragments, 5'-overhangs were filled up by Klenow reaction. DNA polymerase I, large (Klenow) fragment was used according to the manufactures' protocol (1U/ μ g DNA).

To prevent self-ligation of the linearized vector, DNA was dephosphorylated using shrimp alkaline phosphatase to prevent self-ligation.

The ligation of DNA fragments into linearized vector DNA was performed using the T4 DNA ligase in accordance with the manuals' instructions. The dephosphorylated, linearized vector DNA was mixed with restricted insert DNA at a ratio of 1:3 for sticky end ligation or 1:5 for blunt end ligation.

2.3.6 Transformation of competent bacteria

Transformation of chemical competent *E. coli* cells was performed by heat shock. Cells were thawed on ice and 1:1 diluted in KCM solution. 100 μ l of the cell solution were mixed with 10 ng plasmid DNA or 1 μ l ligation mixture. Following heat shock for 45 sec at 42 °C, cells were chilled on ice for 10 min. In the case of ampicillin-resistant plasmids, the transformed cells were directly plated onto LB plates containing 100 μ l/ml ampicillin. In case of kanamycin-resistant plasmids, 1 ml of pre-warmed antibiotic-free LB was added to the cell suspension and the mixture was shaken for 1 h at 37 °C. The cells were centrifuged for 3 min at 1,200 g and the pellet was resuspended in 100 μ l LB. The suspension was plated on LB plates containing 50 μ l/ml kanamycin. The plates were incubated over night at 37 °C.

2.3.7 Plasmid midi preparation

Plasmids for midi preparation were isolated from transformed *E. coli*. A single bacteria colony was inoculated in 200 ml of antibiotic supplemented LB medium and grown over night at 37 °C. The cell suspension was pelleted by centrifugation at 4 °C. The preparation of plasmids was performed using the NucleoBond® Xtra midi kit according to the manuals' instructions. Plasmid concentrations were measured with a Nano Drop 2000c.

2.3.8 Site-directed mutagenesis

The introduction of point mutations in the DNA sequence of a plasmid was generated by PCR, based on the manufacturer's instructions of the Quick Change® Site directed mutagenesis kit (Stratagene). The PCR reaction was set up as follows and incubated in a thermal cycler with the following program.

PCR reaction (50 µl):

5 µl 10x Pfu buffer
 4 µl 2.5 mM dNTP mix
 1,25 µl forward primer (125 ng)
 1,25 µl reverse primer (125 ng)
 1 µl plasmid DNA (12.5 ng)
 0.5 µl *Pfu*Plus! DNA polymerase (2.5 U)
 37 µl ddH₂O

PCR program:

Initial denaturation	95 °C, 1 min	
Denaturation	95 °C, 30 sec	} 18 cycles
Annealing	55 °C, 1 min	
Extension	68 °C, 1 min/kb	
Terminal extension	68 °C, 7 min	

To cleave the template plasmid, the PCR reaction was then digested with DpnI (10 U) for 1 h at 37 °C. 1 µl of digested PCR product was transformed into bacteria.

2.3.9 cDNA synthesis and RT-qPCR

Complementary DNA (cDNA) was synthesized from purified DNA-free RNA samples by reverse transcription. 1 µg total RNA was reverse transcribed using the iScript cDNA synthesis Kit and additional MgCl₂. The reaction mix was incubated in a thermal cycler according to the manufactures' instructions. The cDNA was diluted 1:5 in nuclease-free water. Real time-quantitative PCR (RT-qPCR) was processed in duplicates using the iTaq SYBR Green Supermix with ROX according to the manufactures' protocol. The PCR run was performed on a Step One Plus instrument.

cDNA synthesis reaction (20 µl):

4 µl 5x iScript reaction mix
1 µl 100 mM MgCl₂
1 µl iScript reverse transcriptase
14 µl RNA template

cDNA reaction program:

25 °C, 5 min
42 °C, 30 min
85 °C, 5 min
4 °C, ∞

RT-qPCR reaction (20 µl):

10 µl iTaq SYBR Green supermix
0.5 µl 10 µM forward primer
0.5 µl 10 µM reverse primer
4 µl nuclease-free dH₂O
5 µl cDNA

RT-qPCR program:

95 °C, 3 min
95 °C, 15 sec
60 °C, 30 sec

} 45 cycles

2.4 Biochemical methods

2.4.1 Subcellular fractionation

For the subcellular fractionation of lysates from hippocampal neurons, the cells were washed with ice-cold DPBS and detached in HLB containing protease inhibitors and 0.2 %NP-40 by shaking for 10 min at 4 °C. The cell lysis was achieved through 20 strokes with a Teflon potter. One part of the sample was taken as input and diluted with 10x RIPA buffer to 1x. For nuclear and cytosolic fractions, the lysate was centrifugated at 800 g for 5 min at 4 °C. The supernatant was recovered as cytosolic fraction. The pellet that contains the nuclei was washed once with HLB containing 0.25 M sucrose and dissolved in 1x RIPA (containing protease inhibitors). The input and nuclear sample were lysed by sonication. Subsequently, all fractions were centrifugated by 16,000 g for 10 min at 4 °C and the respective supernatants were recovered as protein extracts. The subcellular fractionation was performed by Peter Störchel.

2.4.2 Protein extraction

For the preparation of whole cell lysates from neurons, cells were washed once with DPBS and resuspended in lysis buffer containing protease inhibitors. BDNF-treated neurons were lysed in lysis buffer additionally containing Phosphatase Inhibitors Cocktail 2 and 3.

2.4.3 Western blot analysis

The protein concentration of cell extracts was determined using the Pierce™ BCA Protein Assay Kit. Equal protein amounts or lysate volumes were mixed with Laemmli sample buffer, boiled for 5 min at 95 °C and loaded onto a 10% SDS-polyacrylamide gel and separated by electrophoresis (SDS-PAGE) in running buffer. Precision Plus Protein Dual Color Standard was loaded as size standard next to the samples. The proteins were then transferred by tank blotting at 90 V and 4 °C for 100 min in blotting buffer to an Amersham Hybond PVDF (poly-vinylidene difluoride) membrane, which was prior activated in 100% methanol. To saturate unspecific binding, the membrane was incubated on a shaker for 1 h with 5% milk powder in TBS-T. The blot was then incubated with a primary antibody (Table 7) diluted in milk containing TBS-T for 2-3 h at RT or overnight at 4 °C. After three times washing for 10 min at RT with TBS-T milk, the blot was incubated with the HRP-conjugated secondary antibody (Table 7) for 1 h at RT, followed by three wash steps with TBS-T. HRP was detected using the ECL Prime Western Blotting Detection Reagent. The band intensities of scanned films were measured with ImageJ software.

2.4.4 Luciferase reporter assay

Luciferase reporter assays were performed by use of the Dual Luciferase Reporter System. Therefore, hippocampal neurons (11 DIV) or cortical neurons (5 DIV, 12 DIV) were transfected in 24 well-plates with equal amounts of firefly (pGL4-3'UTR reporter constructs) and renilla luciferase (pGL4-RL) expressing vectors (Table 3). The luciferase vectors were co-transfected in duplicates as indicated with either pSuper vector (Table 4) or siRNA duplex (Table 2) for knockdown of RBPs, and in the case of miRNA overexpression with miRNA duplex RNA (miR-134 or miR-138) (Table 1). After 2-3 days the cells were washed once in 500 µl DPBS. The DPBS was replaced by 100 µl/well of 1x lysis buffer (Promega) and lysis was carried out by shaking the plate for 20 min at RT. The solutions for the firefly and renilla luciferase measurement were prepared as indicated in the manufactures instructions. After the lysis, 20 µl of each lysate were transferred to a 96-well microtiter plate. The luciferase activity of both firefly and renilla was measured using the GloMax R96 Microplate Luminometer. Firefly activity was normalized to renilla activity and a basal condition which did not contain an shRNA expression vector or an siRNA duplex was set as 1.0 RLA (relative luciferase activity).

Table 8: Vector and RNA duplex quantities used for transfection

Plasmid/ duplex RNA	Amount per well (24-well format)
pGL4-3'UTR	50 – 100 ng
pGL4-RL	50 – 100 ng
pSuper	10 ng
siRNA duplex	7.5 pmol
miRNA duplex	5 pmol

2.4.5 Tethering assay

Tethering experiments were performed by co-transfecting cortical neurons at 5 DIV with 150 ng pCI-Neo-NHA or 250-400 ng NHA-fusion constructs, 50 ng of pRL-5boxB and 50 ng of pGL4.13. The cell lysis and luciferase measurement was performed 2 days after transfection as described in chapter 2.4.4. The renilla activity was normalized to firefly activity.

2.4.6 Co-immunoprecipitation (co-IP)

Co-immunoprecipitation (co-IP) of Ago and Noval was performed in lysates that were prepared from hippocampi of adult female rat brain. The hippocampi of one brain were dissected in ice-cold DPBS and transferred to a pre-chilled 15 ml dounce potter and homogenized on ice by 14 strokes in 2.5 ml lysis buffer containing protease inhibitors. The homogenate was transferred to 2 ml tubes and centrifugated for 10 min at 16,000 g at 4 °C. 50 µl of the supernatant were kept as input, the rest was used for further steps. For RNase treatment RNase A (1:20) was added to one half of the cell lysate. For immunoprecipitation of Ago 500 µl lysate were incubated with mouse anti pan-Ago (1.25 µg). As a control, 500 µl lysate were incubated with mouse IgG (1.25 µg). Antibody incubation occurred in Eppendorf tubes rotating over night at 4 °C. Protein G beads (25 µl per condition) were equilibrated in lysis buffer by rotating for 5 min at 4 °C followed by centrifugation for 2 min at 300 g (twice). Afterwards the beads were blocked for 2 h in BSA diluted in lysis buffer (1 mg/ml). Following two times washing in 1 ml lysis buffer, the beads were resuspended in 30 µl lysis buffer and incubated with lysate and antibody for 1 h at 4 °C followed by four washes in lysis buffer. The last two wash steps contained 350 mM NaCl. After final washing the supernatant was discarded and beads were mixed with sample buffer and prepared for SDS-PAGE.

2.4.7 RNA-immunoprecipitation (RNA-IP)

The hippocampi of an adult female rat were dissected and homogenized in a dounce potter by 20 strokes in 3.5 ml lysis buffer containing complete protease inhibitors. The homogenate was then passed five times through a 26G needle. After centrifugation for 20 min at 70,000 g at 4 °C, the supernatant was supplemented with 100 µg/ml yeast tRNA. Protein G Dynabeads were equilibrated in 1 ml lysis buffer (without inhibitors) by rotating for 5 min at 4 °C. To separate the beads from the solution, the tube was placed on a magnet and the supernatant was removed. The lysate was then precleared with the beads for 30 min at 4 °C. After preclearing, 160 µl of the supernatant were collected as input. The immunoprecipitation was performed by addition of 10 µg antibody (rabbit anti-Noval1(Abcam) or normal rabbit IgG (SCBT)) and 30 µl Protein G Dynabeads to 1 ml of precleared lysate. The solution was rotating for 2 h at 4 °C. Afterwards, the beads were washed five times with lysis buffer (0.1 U/ml SUPERase inhibitors and 10 µg/ml yeast tRNA) and once with lysis buffer without additives. The beads were resuspended in 1 ml lysis buffer and divided to 200 µl (protein) and 800 µl (RNA) fractions. After pelletizing the beads with the magnet, the supernatant was discarded. For protein detection by Western Blot the beads were diluted in sample buffer and prepared for SDS-PAGE. From the other part of the beads RNA was extracted by use of the mirVana™ Isolation Kit. RNA was eluted in 70 µl nuclease-free water followed by digestion of DNA by treatment with Turbo DNase according to the manufacturer's protocol. After denaturation of the DNase for 10 min at 75 °C in the presence of 15 mM EDTA, RNA samples were kept on ice. For detection of specific mRNAs, cDNA was synthesized and RT-qPCR was performed (chapter 2.3.9).

2.5 Immunocytochemistry (ICC)

For microscopic analysis, cultured hippocampal neurons were fixed in 500 µl 4% paraformaldehyde/sucrose in DPBS for 15 min at RT. Subsequently, the cells were washed four times in 500 µl DPBS (2x 30 sec, 2x 5 min). For the analysis of dendritic spines and dendritic growth, the GFP expressing cells were mounted on microscope slides in Aqua-Poly/Mount. For immunocytochemistry the coverslips were transferred to a wet chamber. Primary and secondary antibodies were diluted in GDB (gelatine detergent buffer). The cells were incubated with the primary antibody for 1 h at RT in the dark.

After four wash steps with DPBS (1x 30 sec, 3x 5 min) the secondary antibody was applied for 1 h at RT protected from light. After three further wash steps with DPBS, Hoechst was incubated at 1:5000 in DPBS for 5 min. After all, the cells were mounted as described above (chapter 2.5).

2.6 Microscopic analysis

All microscopic images were taken with a Confocal Laser Scanning microscope. The presented immunocytochemistry images are the maximum projections of 2 - 4 confocal stacks with a z -distance of 0.5 – 1 μm . The measurements of average signal intensity and x - y -distance as well as further image processing were carried out with the ImageJ software.

2.7 Spine assay

For dendritic spine analysis, hippocampal neurons (13 DIV) were co-transfected with 200 ng pEGFP-Amp, 5 ng of shRNA expressing pSuper construct and miR-134 duplex RNA. After 6 days (19 DIV) the cells were prepared for confocal imaging (chapter 2.5). Confocal z -stack images were taken with a 63x objective (7 z -stacks at 0.4 μm interval) and projected to a single plane image. The individual spine size was quantified by its mean grey value in a 2.2 μm^2 -circle using ImageJ software. More than 100 spines per cell were measured and normalized to the cell's mean grey values. Spines from three independent experiments were measured. The imaging and analysis were carried out blinded to the experimental conditions. The spine assay was performed by Anna Tsankova under supervision of Peter Störchel.

2.8 Sholl analysis

For the determination of dendritic complexity by Sholl analysis, pyramidal neurons (12 DIV) expressing GFP were imaged by fluorescence microscopy. The images were layered with a pattern of concentric circles with increasing radii, centered to the cell body (8 circles with distances from soma between 25 – 200 μm). The dendritic outgrowth was determined by quantification of intersections between dendrites and each circle. The sum

of all dendrite intersections at each distance represents the total number of intersections. Per condition 10 to 12 cells were analyzed.

2.9 Statistical analysis

Statistical analysis for pairwise comparison was carried out by two-tailed, unpaired Student's t-test. For multiple conditions, one-way Anova test (<http://vassarstats.net/>) was applied with Tukey HSD post-test comparing single conditions pairwise. Significance was set at $p < 0.05$.

3 Results

3.1 Background – RNAi-based screen

Previously, a large-scale RNAi-based screen in primary mouse cortical neuron cultures was performed in our laboratory to identify novel neuronal RBPs that modulate miRNA activity (Gabriele Siegel, PhD thesis, 2011). The study was based on the idea that the knockdown of crucial effector proteins should relieve the miRNA-mediated repression of translation that can be monitored by a luciferase reporter assay. The luciferase reporter construct contained downstream of the luciferase coding sequence the 3'UTR of the gene *Ube3a* which is responsive to the neuronal miRNA miR-134 (Valluy *et al.* 2015). A subset of 286 RBPs that are expressed in the postnatal mouse cortex were chosen as candidate proteins (McKee *et al.* 2005). In the screening experiment each of the candidates was individually knocked down by transfection of synthetic siRNAs. Specifically, three different siRNAs for each RBP were separately co-transfected with the *Ube3a* luciferase reporter and miR-134-duplex RNA into primary mouse cortical neurons (5 days in vitro (DIV)). After three days the luciferase activity was measured (Figure 5 A).

Transfection of miR-134 duplex RNA led to an about 40% repression of the *Ube3a*-luciferase reporter expression. In the case that an RBP was required for the inhibitory function of miR-134, we expected that the reporter repression was relieved in the presence of the respective siRNAs. Candidates were considered as positive hits if at least two out of three siRNAs could relieve the reporter repression by at least 50%. Using this criteria, 12 RBPs that are required for the function of miR-134 were identified in this study (Figure 5 B). Among the positive hits were *Tnrc6c* and *Ddx6*, two proteins that are known to be directly associated with miRISC (Chu *et al.* 2006; Chen *et al.* 2009). This confirmed that the screen setup allowed the identification of miRISC-associated factors. Importantly, ten new candidates (*Ewsr1*, *Fubp1*, *Lsm7*, *Ncoa3*, *Nova1*, *Nxf1*, *Nxt1*, *Rbm25*, *Rpl5* and *U2af1*) were detected that had not yet been described in the context of miRISC regulation (Figure 5 B). A miRNA-independent function in RNA metabolism was previously reported for most of these RBPs. For example, *Nova1*, *U2af1* and *Rbm25* are involved in the regulation of RNA splicing (Jensen *et al.* 2000; Mollet *et al.* 2006; Zhou *et al.* 2008)

while others influence mRNA translation (Rpl5) (Meskauskas *et al.* 2001) or mRNA decay (Lsm7) (Tharun *et al.* 2000). The RBPs Ewsr1, Fubp1 and Ncoa3 had been implicated in transcription, whereas Nxf1 and Nxt1 were shown to regulate the nuclear export of mRNA.

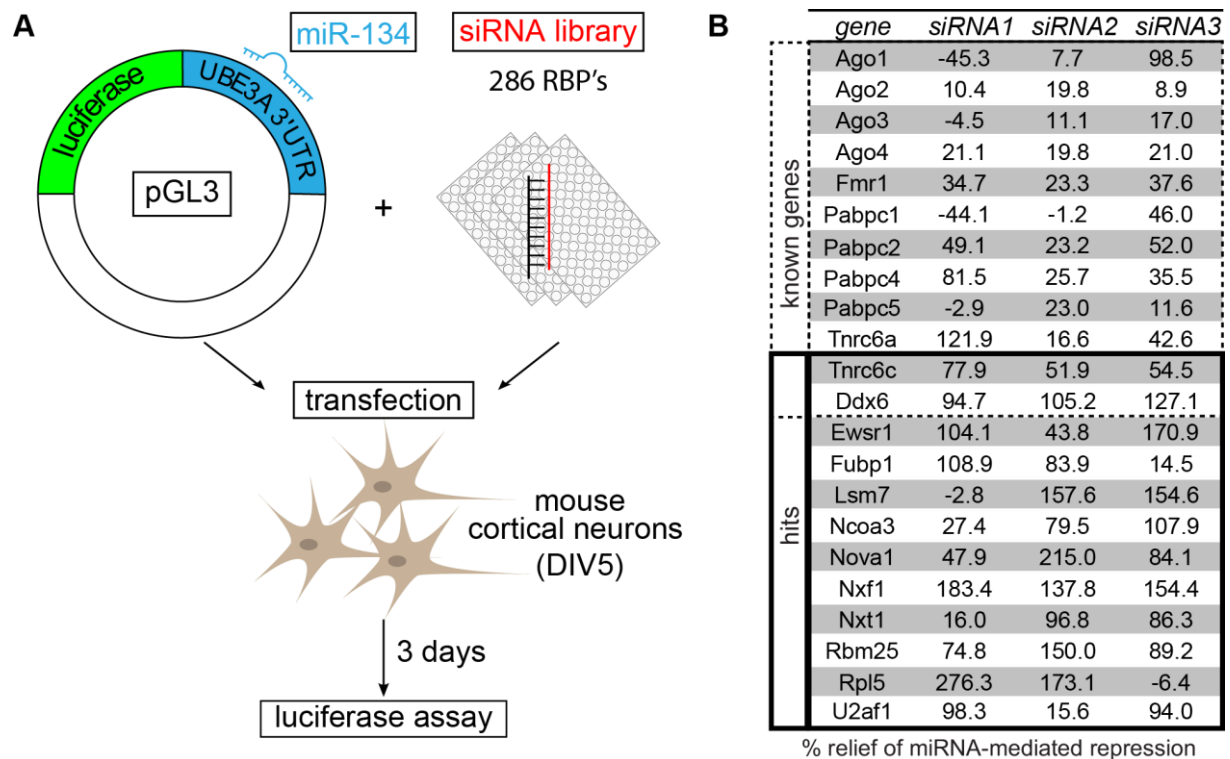


Figure 5: Large scale RNAi-based screen in primary mouse neurons

A) Schematic overview of the RNAi screen in mouse primary neurons performed by G. Siegel. Cortical Neurons (5 days in vitro (DIV)) were co-transfected with pGL3-Ube3a 3'UTR luciferase reporter, miR-134 duplex RNA and one siRNA targeting individual RBPs. Three different siRNAs were used for every RBP. After three days of incubation luciferase activity was determined. **B)** List of a representative subset of genes that were tested in the screen. The dashed line designates "known genes" that are involved in the regulation of miRNA activity. Thick line marks candidate genes that relieved reporter repression by at least 50% with at least two out of three tested siRNAs. Values represent the relief of miRNA-mediated repression after siRNA treatment, obtained from three independent experiments.

3.2 Validation of RNAi screen results

For the validation of the RNAi-based screen results (Siegel, 2011) I performed a secondary screen using only candidates that were classified as positive hits in the first study. To exclude false positives, the experimental setup was modified in the following points.

First, I used an improved luciferase reporter system that should reduce potential non-specific siRNA effects. The Ube3a 3'UTR was cloned downstream of the firefly coding sequence of the pGL4 plasmid (UBE3A-luc). Compared to the previously used pGL3 vector, pGL4 contains less regulatory elements in the vector backbone. Thus, it minimizes the possibility of non-specific siRNA effects caused by factors that can potentially interact with regulatory elements in the vector. The UBE3A-luc reporter was co-transfected with the miR-134 duplex RNA and the siRNAs for specific RBPs individually into immature rat cortical neurons (5 DIV). Furthermore, a condition was included where the reporter was co-transfected with the siRNA in the absence of miR-134 duplex. This condition should control for possible non-specific effects on luciferase mRNA translation caused by the siRNAs. As a further control, an empty luciferase vector lacking 3'UTR sequence was co-transfected with the siRNA in the presence or absence of miR-134 duplex. In the secondary screen only nine out of the 12 positive candidates (Nova1, Ncoa3, Ewsr1, Tnrc6c, Rpl5, Nxt1, Rbm25, U2af1, and Nxf1) were tested. Since the secondary screen was performed in rat, an siRNA was selected for each candidate that targets a sequence conserved between rat and mouse genomes and showed high efficacy in the primary screen. *Lsm7* and *Fubp1* were excluded, as none of the respective siRNAs was conserved between rat and mouse. Furthermore, *Ddx6* was not included in the secondary screen since it was already shown to be involved in the regulation of miRNA activity (Chu *et al.* 2006). The known miRISC protein *Tnrc6c* was used as positive and a scrambled siRNA nucleotide (control siRNA) as a negative control in the screen. Luciferase activity was measured three days after transfection (8 DIV) and the experiment was performed three times.

The results are presented as ratio between the condition that contains the miR-134 duplex RNA (“+miR”) and the condition without the miR-134 duplex RNA (“-miR”). In the basal condition where no siRNA was co-transfected as well as in the condition with control siRNA the ratio of “+miR” to “-miR” was about 0.5 (0.53 ± 0.05 (basal); $0.51 \pm$

0.05 (control siRNA)) (Figure 6 A). This reflects a nearly 50% downregulation of the UBE3A-luc reporter expression mediated by miR-134 overexpression. Compared to control siRNA (0.51 ± 0.05) the downregulation was significantly relieved when siRNAs against Nova1 (0.87 ± 0.20), Ncoa3 (0.84 ± 0.16), Ewsr1 (0.69 ± 0.08) and Tnrc6c (0.73 ± 0.14) were co-transfected. This suggests that Nova1, Ncoa3 and Ewsr1 are partially required for the repressive function of miR-134, in a comparable manner to Tnrc6c. Surprisingly the siRNAs against Rpl5 (0.62 ± 0.27), Nxt1 (0.52 ± 0.20), Rbm25 (0.51 ± 0.07), U2af1 (0.49 ± 0.05) and Nxf1 (0.46 ± 0.03) did not significantly change the ratio “+miR” to “-miR” in the UBE3A-luc reporter. This suggests that knockdown of these RBPs might affect reporter gene expression independent of miR-134, for example due to a general role of these RBPs in mRNA translation. None of the siRNAs had a significant effect on the empty luciferase vector demonstrating that the effects of the siRNAs were strictly dependent on the presence of 3'UTR regulatory sequences (Figure 6 B).

Among all candidates, the knockdown of Nova1 had the strongest activating effect on reporter gene expression. We therefore decided to study this RBP in more detail. The focus of this thesis was to characterize the role of Nova1 in the regulation of miRNA function and to understand the molecular mechanism whereby Nova1 knockdown impairs miRNA mediated inhibition of target mRNAs.

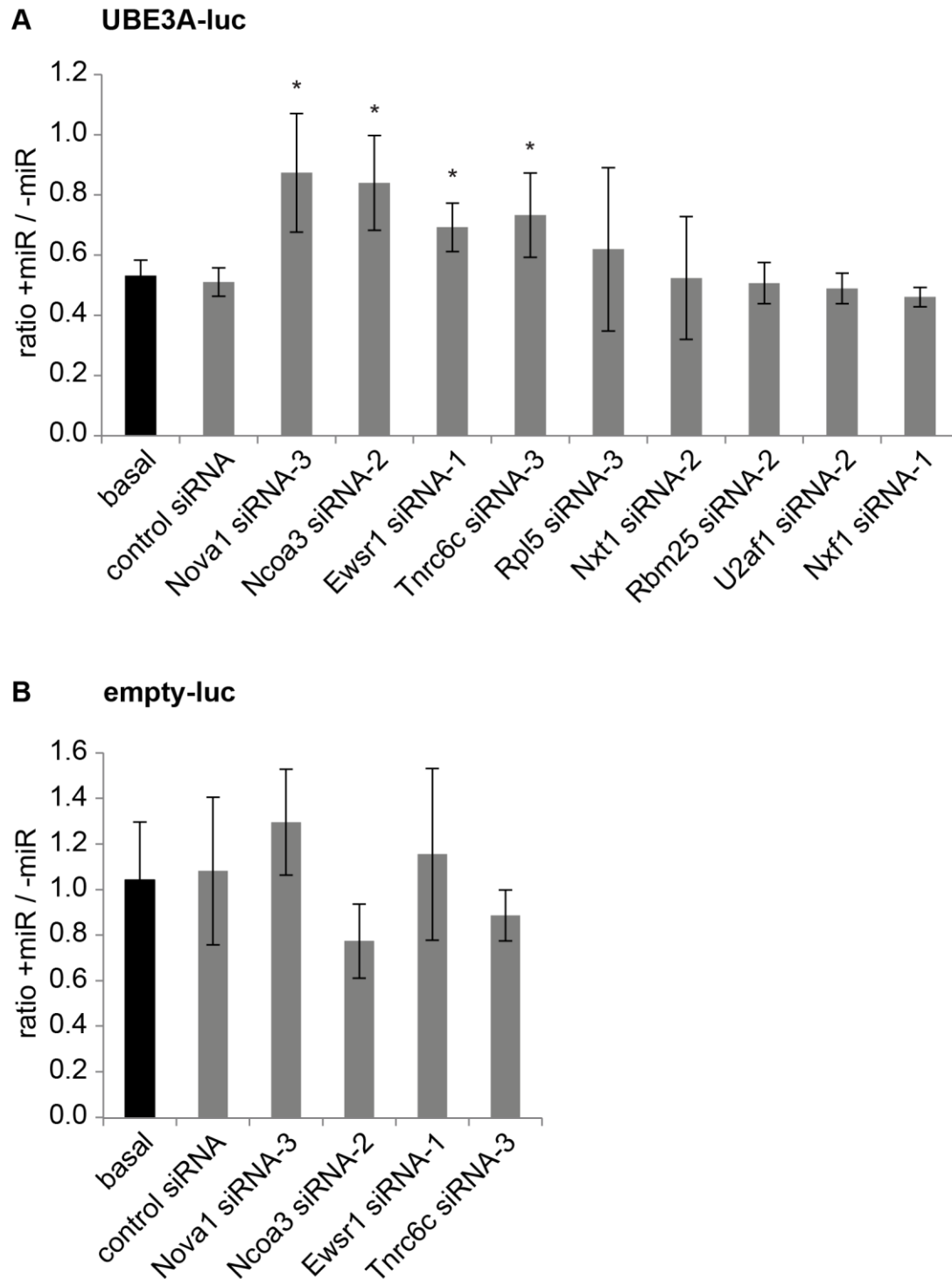


Figure 6: Luciferase reporter assay for the validation of positive hits using RNAi

A) The pGL4-Ube3a-3'UTR luciferase reporter (UBE3A-luc) was co-transfected with miR-134 duplex RNA and one of the indicated siRNAs into rat cortical neurons at 5 DIV. Numbers of siRNAs refer to their original designation in the first screen. Plotted are normalized luciferase values as ratio of a condition with miR-134 duplex ("miR") to a condition without miR-134 duplex ("no-miR"). Values are the average from three independent experiments \pm standard deviation. *, $p < 0.05$ (unpaired Student's t-test compared to control siRNA). **B)** An empty pGL4 plasmid was co-transfected together with miR-134 duplex RNA and one of the indicated siRNAs into rat cortical neurons at 5 DIV. Values are plotted as in (A). *, $p < 0.05$ (unpaired Student's t-test compared to control siRNA)

3.3 Expression analysis of Nova1 in primary neuronal cultures

The endogenous expression of Nova1 protein was examined in cultured neurons using Western blot analysis and immunocytochemistry. First, protein lysates of rat hippocampal and cortical neurons were prepared at five different time points ranging from 7 DIV to 17 DIV (hippocampus) or 5 DIV to 14 DIV (cortex). As shown in figure 7 A and B, Nova1 was detected at all time points in both cell types, suggesting that it is expressed throughout the *in vitro* neuronal development. Expression of β -actin in the same lysates served as a loading control.

In the next step, the subcellular localization of Nova1 protein was analyzed in more detail by immunocytochemistry (ICC) of hippocampal neurons (14 DIV) with an anti-Nova1 antibody. The neuronal cell morphology was visualized by staining for the microtubule-associated protein 2 (MAP2, green). The nuclei were stained with Hoechst dye (blue). Nova1 signal, shown in red, was observed in the cytoplasm and nucleus (Figure 7 C). The majority of cytoplasmic Nova1 was detected as granules surrounding the nucleus. Few Nova1 positive granules were also observed in the proximal parts of dendrites (arrows Figure 7 D).

The subcellular localization of Nova1 assessed by ICC could be confirmed by a Western blot analysis of cytoplasmic and nuclear fractions prepared from rat cortical neurons. Nova1 was detected in both fractions, with a more prominent expression in the nuclear fraction (Figure 7 E). Western Blots for α -tubulin and HDAC2 (Histone Deacetylase 2) confirmed the proper separation of cytoplasmic and nuclear fractions in this experiment.

In conclusion, Nova1 protein is expressed in both the nucleus and cytoplasm of hippocampal neurons during the neuronal development between 7 and 14 DIV.

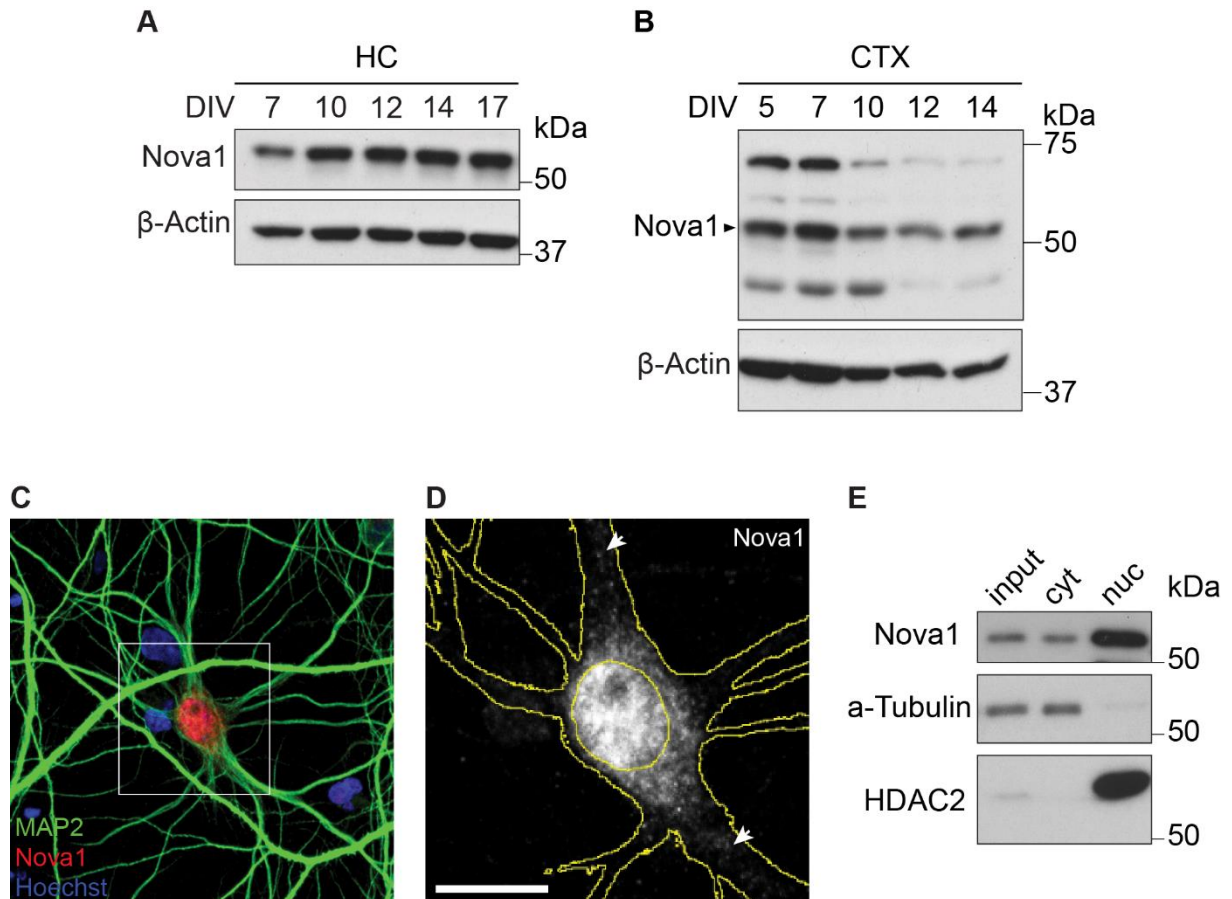


Figure 7: Expression of Nova1 in primary rat neurons in culture

A), B) Western blot analysis of whole-cell protein lysates prepared from hippocampal (HC) (**A**) or cortical (CTX) (**B**) neuronal cultures at the indicated days *in vitro* (DIV). β -actin was used as a loading control. **C), D)** Immunocytochemistry analysis of Nova1 expression in hippocampal culture at 14 DIV. **C)** Signal of Nova1-specific antibody is pictured in red. The neuronal cell was visualized by a Map2 staining (green). Nuclei were stained with Hoechst (blue). **D)** Magnification of the insert depicted in (**C**) shows Nova1 signal in grayscale and nuclear and cellular outlines in yellow. Scale bar = 10 μ m. **E)** Western blot analysis of Nova1 of whole cell lysates (input) and cytoplasmic (cyt) or nuclear (nuc) fractions obtained from FUDR-treated rat hippocampal neurons at 7 DIV. α -tubulin was used as cytoplasmic marker. HDAC2 was used as nuclear marker. The fractionation experiment was performed in contribution with Peter Störchel.

3.4 Nova1 is required for the repressive function of miRNAs

3.4.1 Nova1 knockdown impairs miR-138 repressive activity in young cortical neurons

To characterize the specific role of Nova1 in the regulation of miRNAs it was of interest to investigate whether Nova1 is required for the repressive function of other miRNAs apart from miR-134. To address this point, I examined the activity of the neuronal miRNA miR-138 in the context of Nova1 knockdown using luciferase reporter gene assays.

Luciferase assay was carried out using the miR-138 responsive APT1 3'UTR luciferase construct in the pGL4 vector (APT1-luc). APT1 (Acyl-Protein-Thioesterase-1) is encoded by the *LYPLA1* gene which contains a binding site for miR-138 in the 3'UTR (Siegel *et al.* 2009).

Analogous to the secondary screen (chapter 3.2) the luciferase reporter was co-transfected into young rat cortical neurons (5 DIV) together with the miR-138 duplex RNA and either control siRNA or the siRNAs targeting *Nova1* or *Ewsr1*, respectively. The luciferase activity was measured three days after transfection.

The results are presented as ratio between the condition with miR-138 duplex RNA (“+miR”) and the condition without miR-138 duplex RNA (“-miR”) (Figure 8). Overexpression of miR-138 only (basal) led to a significant repression of the APT1 luciferase reporter (0.34 ± 0.07). The knockdown of *Nova1* caused a significant relief of the repression (0.76 ± 0.26) whereas the knockdown of *Ewsr1* had no effect on the reporter expression (0.32 ± 0.07). This result suggests that *Nova1* knockdown interferes with miR-138 mediated repression of reporter gene expression similar to miR-134 function. In conclusion, this suggests that *Nova1* regulates the activity of several neuronal miRNAs, whereas *Ewsr1* might be more specific for the regulation of miR-134.

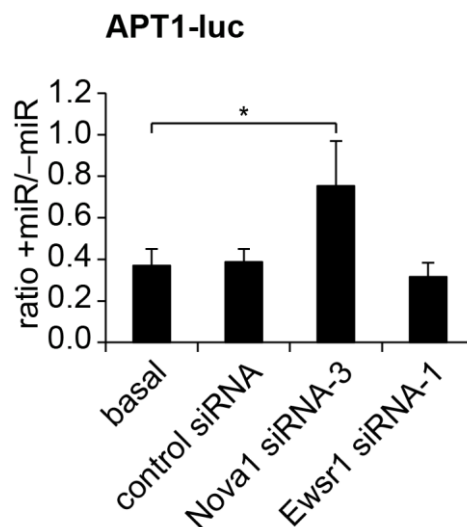


Figure 8: Luciferase reporter assay of APT1-luc in cortical neurons overexpressing miR-138

pGL4-APT1 3'UTR (APT1-luc) was co-transfected into rat cortical neurons at 5 days *in vitro* (DIV) with indicated siRNAs in the presence (“+miR”) or absence (“-miR”) of miR-138 duplex RNA. Values represent ratio of the two conditions (“+miR”/“-miR”) and are the average from three independent experiments ± standard deviation. *, $p < 0.05$ (unpaired Student's t-test compared to control siRNA).

3.4.2 Nova1 is required for the repressive activity of endogenous miR-134 and miR-138

Nova1 mediates the repressive activity of overexpressed miR-134 and miR-138, and Nova1 may also be necessary for the activity of endogenously expressed miRNAs in neurons. In order to answer this question, luciferase assays were performed in rat cortical neurons at 14 DIV, as miRNAs are abundantly expressed at this developmental stage (Schratt *et al.* 2006; Siegel *et al.* 2009). The 3'UTR luciferase reporters, APT1-luc and UBE3A-luc, were transfected into cortical neurons two days earlier (12 DIV). Knockdown of Nova1 was achieved with an shRNA expression vector (pSuper) that gives rise to the previously used Nova1 siRNA-3 (chapter 3.2 and 3.4.1). To validate the specificity of the Nova1 shRNA a rescue experiment was performed, expressing an shRNA-resistant GFP-Nova1-fusion protein (GFP-Nova1^R) in the context of the Nova1 shRNA. The results are presented as relative luciferase activity (RLA) values that were normalized to basal condition, expressing the reporter plasmid APT1-luc or UBE3A-luc only.

The knockdown of Nova1 significantly increased the expression of APT1-luc (1.40 ± 0.18) and Ube3a-luc (1.29 ± 0.18) compared to the control shRNA (0.78 ± 0.15 (APT1-luc); 0.83 ± 0.06 (Ube3a-luc)) (Figure 9 A, B). This effect is in agreement with the Nova1 siRNA-mediated relief of repression of the luciferase reporter (Figure 6 A and 8). Reintroduction of Nova1 by co-transfection of the shRNA-resistant GFP- Nova1^R construct partially rescued the effect of the Nova1 shRNA, since APT1-luc (1.09 ± 0.10) and UBE3A-luc (1.00 ± 0.27) expression was significantly decreased. In contrast, expressing GFP only (1.41 ± 0.09 (APT1-luc); 1.27 ± 0.15 (Ube3a-luc)) did not alter the effect of the Nova1 shRNA which confirms that changes in Nova1 protein levels are responsible for the observed differences of luciferase expression. In sum, these results suggest that Nova1 is required for the repression of reporter genes that are regulated by the endogenous miR-134 and miR-138 in rat cortical neurons. In addition, overexpression of GPF-Nova1 significantly decreased the expression of the APT1-luc as well as the UBE3A-luc reporter compared to the GFP control (Figure 9 C and D). These results suggest that Nova1 is both necessary and sufficient to repress Ube3a and APT1 expression in neurons.

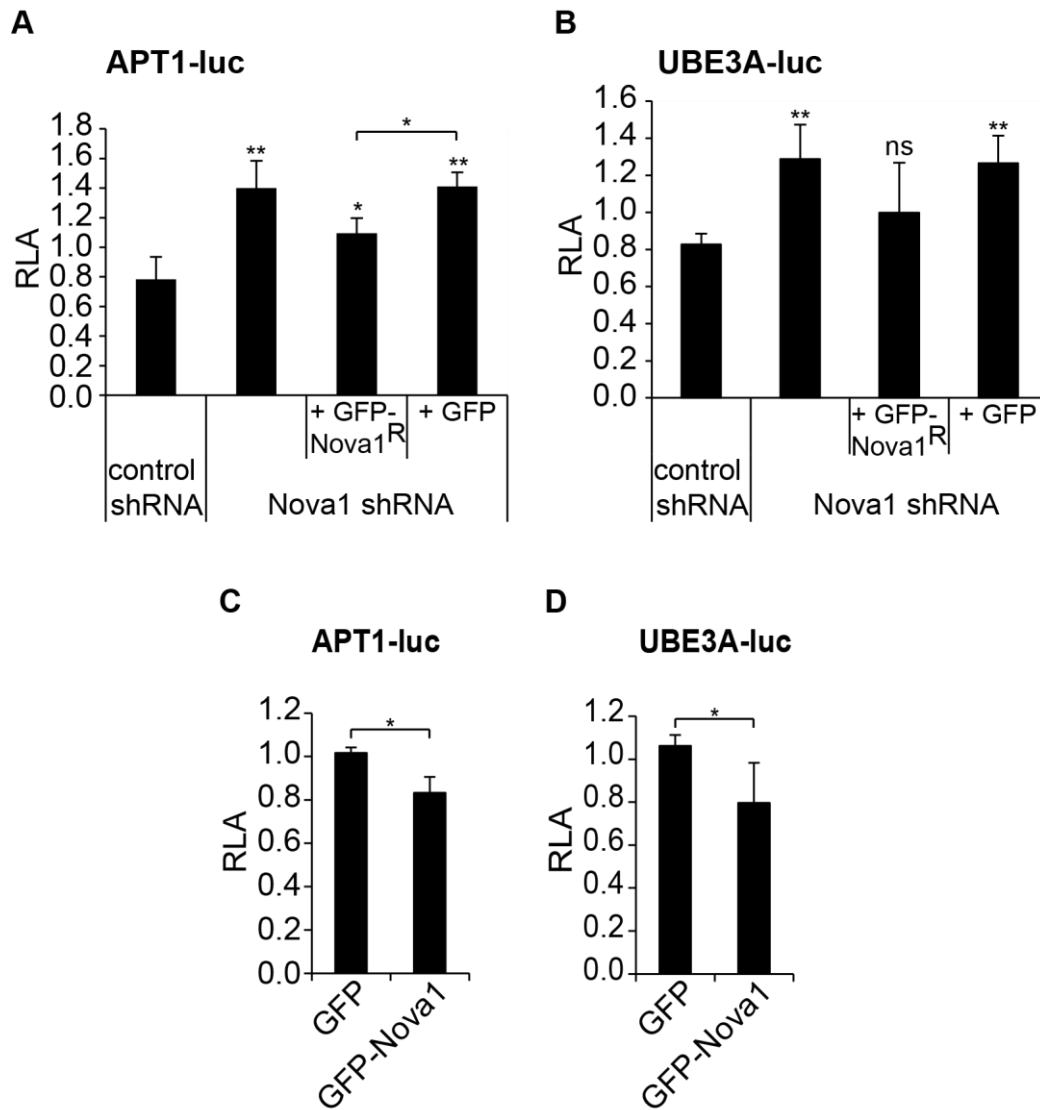


Figure 9: Luciferase reporter assays of APT1-luc and Ube3a-luc in mature cortical neurons

A-D) Luciferase assay in rat cortical neurons (14 DIV) that had been transfected with pGL4-APT1-3'UTR (APT1-luc) or pGL4-UBE3A-3'UTR (UBE3A-luc). In A) and B) pSuper vectors expressing either Noval shRNA or control shRNA and a shRNA resistant GFP-Noval fusion construct or a GFP control vector were co-transfected. In C) and D) a GFP-Noval fusion construct or GFP control vector were co-transfected. Relative luciferase activity (RLA) represents the ratio of firefly reporter to renilla control reporter normalized to a basal condition without shRNA expressing vector (A, B) or 3'UTR luciferase reporter only (C, D). Values are the average from three independent experiments \pm standard deviation. *, $p < 0.05$ (unpaired Student's t-test compared to control shRNA or as indicated).

3.4.3 Nova1 knockdown interferes with miR-134-, miR-124- and let-7-dependent repression of luciferase reporter genes

In order to verify that the repressive function of Nova1 indeed depends on miRNA activity a set of luciferase reporters with mutated miRNA binding sites was generated by site directed mutagenesis. The set consists of luciferase reporters containing 3'UTRs of *Limk1* (LIMK1-luc), a target gene of miR-134; *Iqgap1* (IQGAP1-luc), a target gene of miR-124; as well as *lin41* (LIN41-luc) and *HMGA2* (HMGA2-luc), two target genes of the miRNA let-7. In addition, mutant reporters with disrupted miRNA binding sites were generated for all mentioned 3'UTRs. The constructs were individually co-transfected into mature cortical neurons at 12 DIV together with the Nova1 shRNA or control shRNA vector. The results are displayed as relative luciferase activity (RLA), normalized to a basal condition which contains the respective reporter plasmid without shRNA expressing vector.

The LIMK1 wildtype reporter (LIMK1 WT) exhibits a significant increase of luciferase activity under Nova1 knockdown condition (1.63 ± 0.14) (Figure 10 A) similar to the effects that were observed for APT1-luc and UBE3A-luc (Figure 9). Activity of LIMK WT was significantly higher compared to LIMK1 mut (1.34 ± 0.10) upon Nova1 knockdown (Figure 10 A). Similar observations were made for the reporter IQGAP1-luc. Under Nova1 shRNA condition the expression of IQGAP1 WT (1.63 ± 0.13) was significantly increased compared to IQGAP1 mutant (1.23 ± 0.03) (Figure 10 B). Likewise, the luciferase activity of the let-7 target gene *lin41* (Vella *et al.* 2004) (LIN41-luc) revealed a significant difference between wildtype (LIN41 WT) and mutant (LIN41 mut) specifically under Nova1 knockdown conditions (1.84 ± 0.26 (WT); 1.24 ± 0.15 (mut)) (Figure 10 C). In contrast the wildtype (HMGA2 WT) and mutant (HMGA2 mut) reporter of another let-7 target gene, *HMGA2* (Nishino *et al.* 2008), were both significantly upregulated to a similar extent under Nova1 knockdown condition (1.46 ± 0.20 (WT); 1.35 ± 0.14 (mut)) compared to the shRNA control condition (Figure 10 D). Taken together these results suggest that Nova1 represses the LIMK1-, IQGAP1- and LIN41- luciferase reporters in a manner dependent on the validated miRNA binding sites. In contrast, Nova1 regulation of HMGA2 appears to be let-7-independent and may therefore depend on other miRNA binding sites present in the HMGA2 3'UTR.

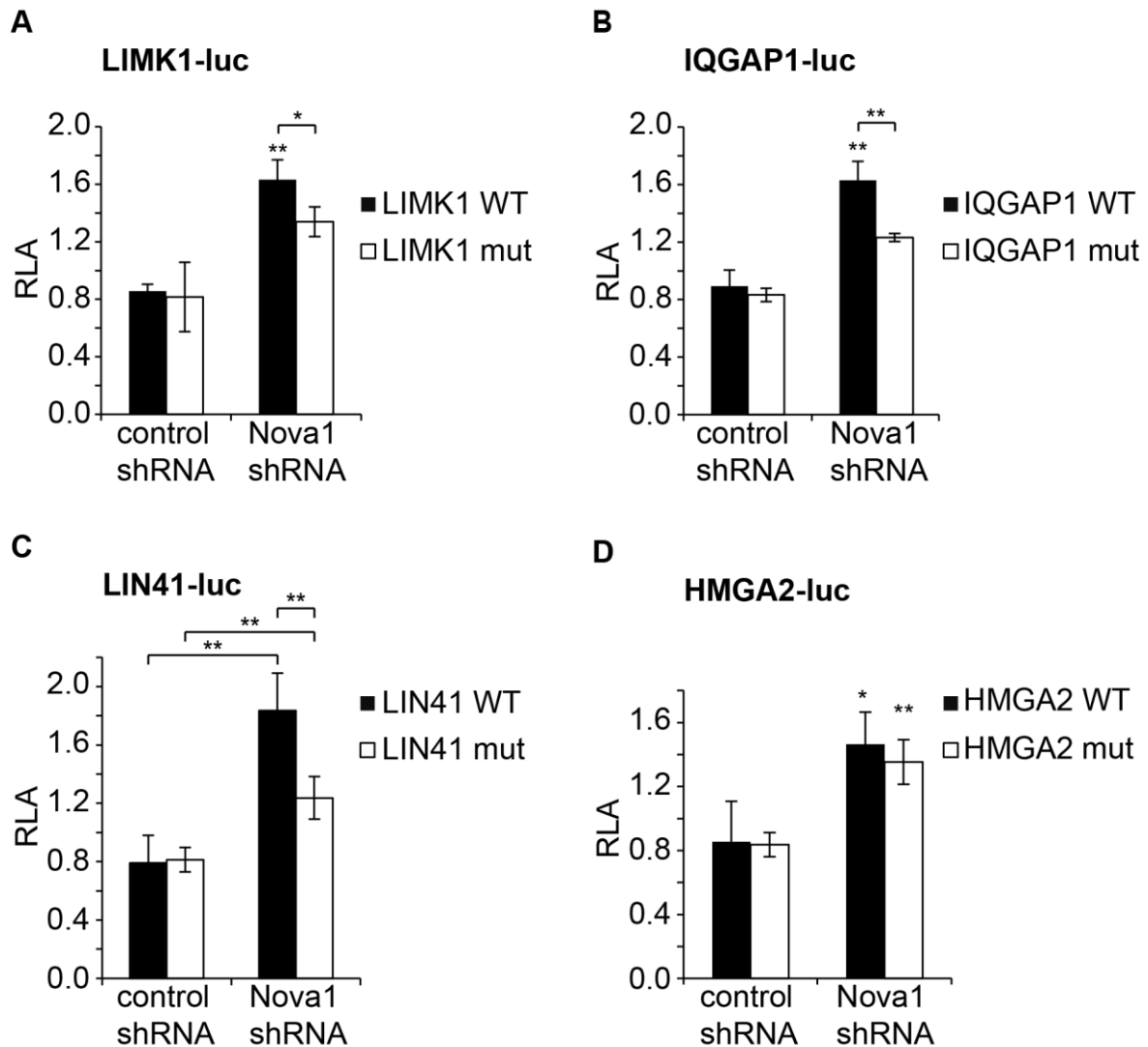


Figure 10: Luciferase reporter assays of wildtype and mutated miRNA binding site reporter in mature cortical neurons

A-D) Luciferase assay in rat cortical neurons (14 DIV) that had been transfected with the indicated 3'UTR luciferase constructs at 12 DIV together with pSuper vectors expressing either Nova1 shRNA or control shRNA. Relative luciferase activity (RLA) represents the ratio of firefly reporter to renilla control reporter normalized to a basal condition without shRNA expression vector. Values represent averages from at least three independent experiments \pm standard deviation. *, $p < 0.05$ **, $p < 0.01$ (unpaired Student's t-test compared to control shRNA or as indicated by bars).

3.4.4 Nova1-dependent regulation of miRNA activity is independent of the 3'UTR context

The previous results demonstrate that Nova1 acts in a miRNA-dependent manner on the repression of numerous target gene reporters. Next, I analyzed whether the presence of a miRNA binding site alone is sufficient for the regulatory function of Nova1. In order to examine isolated miRNA-binding sites, I performed luciferase assays with a miR-138

specific sponge reporter. The miR-138 sponge construct (138sponge luc) harbors six consecutive binding sites for miR-138 downstream of the firefly coding sequence, but lacks other 3'UTR sequences (Figure 11 A). This sponge construct provides a suitable tool to examine whether regulatory sequence elements outside miRNA binding sites are required for the function of Nova1. A construct in which the miR-138 binding site array was cloned in reverse orientation (ctrl sponge) served as a control. The expression of 138sponge luc was significantly increased under Nova1 shRNA condition (1.52 ± 0.18) compared to the control shRNA condition (0.98 ± 0.12) (Figure 11 B). In contrast, the control sponge reporter expression did not change significantly upon Nova1 knockdown (1.11 ± 0.18) compared to the control shRNA (0.93 ± 0.03). This result indicates that Nova1 can act as a regulator of miRNA function independently of the 3'UTR sequence context.

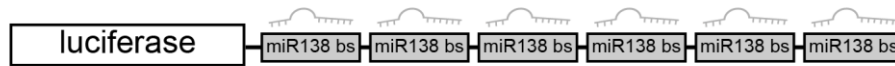
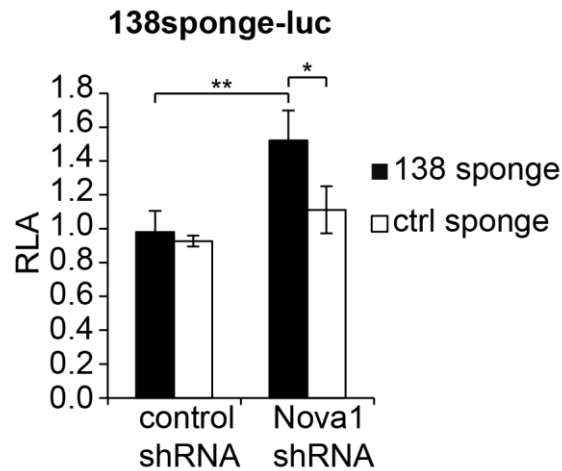
A**B**

Figure 11: Luciferase reporter assay of miR-138 sponge reporter in mature cortical neurons

A) Illustration of the miR-138 sponge luciferase reporter construct (138sponge luc). Six sequential miR-138 binding sites are located downstream of the luciferase coding sequence in place of an entire 3'UTR sequence. **B)** Luciferase assay in rat cortical neurons (14 DIV) transfected with the miR-138 sponge luciferase construct (138 sponge) or a control containing the reversed sequence of the 138 sponge (ctrl sponge) together with pSuper vectors expressing either Nova1 shRNA or control shRNA. Relative luciferase activity (RLA) represents the ratio of firefly reporter to renilla control reporter normalized to a basal condition without shRNA expression vector. Values represent averages from at least three independent experiments \pm standard deviation. *, $p < 0.05$ **, $p < 0.01$ (unpaired Student's t-test).

3.4.5 Nova1 is required for miR-134 mediated repression of endogenous Limk1

MiRNA-mediated mRNA repression usually leads to decreased protein expression. Consequently, it was of interest to study whether the observed regulation of artificial miRNA target gene reporters by Nova1 corresponds to alterations in the respective endogenous protein levels. In order to yield a high Nova1 knockdown efficiency in the neuronal culture, shRNAs were delivered via infection of rAAV particles. Therefore, whole cell lysates were obtained from cortical neurons at 14 DIV that had been infected with recombinant adeno-associated virus (rAAV) expressing Nova1 shRNA. Nova1 knockdown efficiency was assessed by Western blot using lysates from rAAV-Nova1 shRNA infected neurons. Nova1 levels were significantly reduced in Nova1 shRNA compared to control shRNA infected neurons (Figure 12 A).

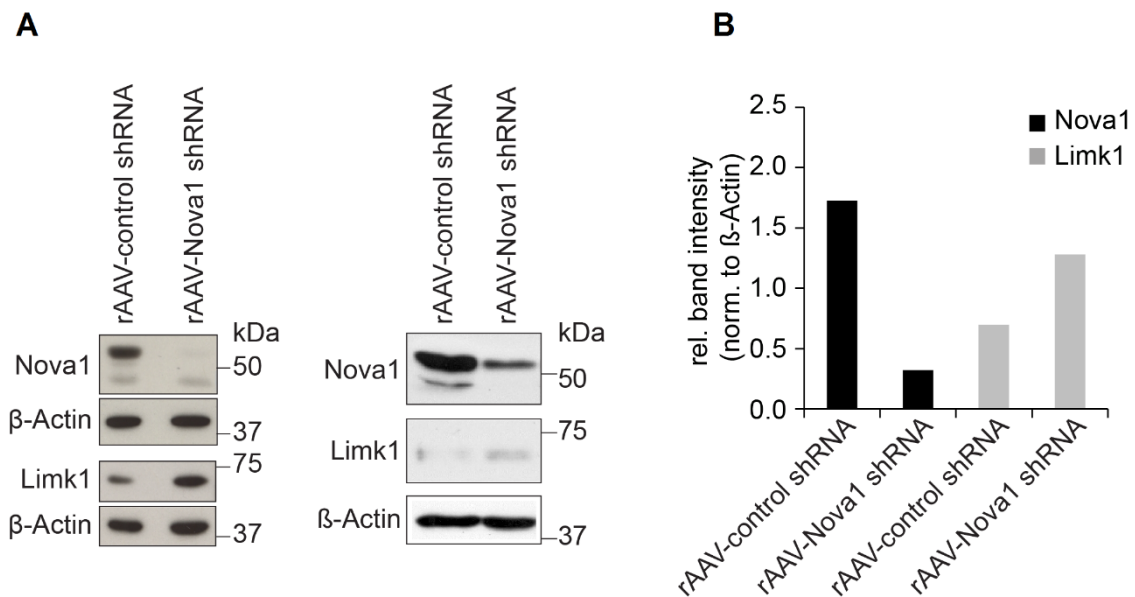


Figure 12: Western blot analysis upon rAAV-mediated Nova1 knockdown

A) Western blot analysis of Nova1 and Limk1 protein in 14 DIV old cortical neurons that had been infected with rAAV expressing either Nova1 shRNA or control shRNA. β -Actin served as a loading control. Results of two independent experiments are shown. **B)** Quantification of Western blot signal intensity. Bars represent relative band intensities that were normalized to β -Actin of the two independent experiments shown in A.

For the analysis of miRNA target gene expression, I focused on the miR-134 target Limk1 which is an important regulator of spine morphogenesis in dendrites. Limk1 protein amounts were increased by a factor of 1.8 in lysates obtained from two independent

infections of neurons with Nova1 shRNA compared to control shRNA as visualized by band quantification and normalization (Figure 12). In conclusion, Nova1 is required for the repression of the endogenous miRNA target protein Limk1. This result is consistent with the observation that Nova1 knockdown significantly upregulates the expression of the Limk1 luciferase reporter in a miR-134 - dependent manner (Figure 10 A).

3.5 Nova1 interaction studies

3.5.1 Nova1 interacts with Ago in lysates obtained from the rat hippocampus

The previous results imply that Nova1 regulates the repression of several miRNA targets irrespective of the 3'UTR context (Figure 10 and Figure 11). I therefore hypothesized that Nova1 could directly interact with miRISC proteins, rather than with the miRNA target mRNA. A co-Immunoprecipitation (co-IP) experiment was performed in hippocampal lysates, obtained from adult rat brain, to test a putative interaction between Nova1 and Ago, the major component of miRISC. Ago proteins were precipitated with a specific pan-Ago antibody and detected by Western blot (Figure 13 A). In the upper part of the figure a specific enrichment of Ago2 in the Ago IP compared to the IgG control can be observed. This demonstrates successful precipitation of Ago2 with the pan-Ago antibody. Ago2 was not detectable in the input lysate, probably due to a low solubilization of Ago2 achieved with this lysis protocol. Importantly, a band migrating at the expected molecular weight of Nova1 could be specifically detected in the pan-Ago-IP fraction but not in the IgG control. This result suggests that Nova1 and Ago2 interact in the rat hippocampus. In conclusion, Nova1 represents a novel interactor of neuronal miRISC.

Performing additional co-IP experiments, I investigated whether an association between Ago2 and Nova1 protein was dependent on the presence of RNA. For this purpose, lysates were treated with RNase before immunoprecipitation. The specific enrichment of Ago2 in pan-Ago immunoprecipitates was again confirmed by Western blot as presented in the upper part of Figure 13 B. Similar to Figure 13 A, a band at the molecular size of Nova1 was observed by anti-Nova1 Western blot in the pan-Ago IP in the presence of RNase. This result suggests that the interaction between Nova1 and Ago does not require the presence of intact RNA, which further implies that Nova1 and Ago reside within a protein complex.

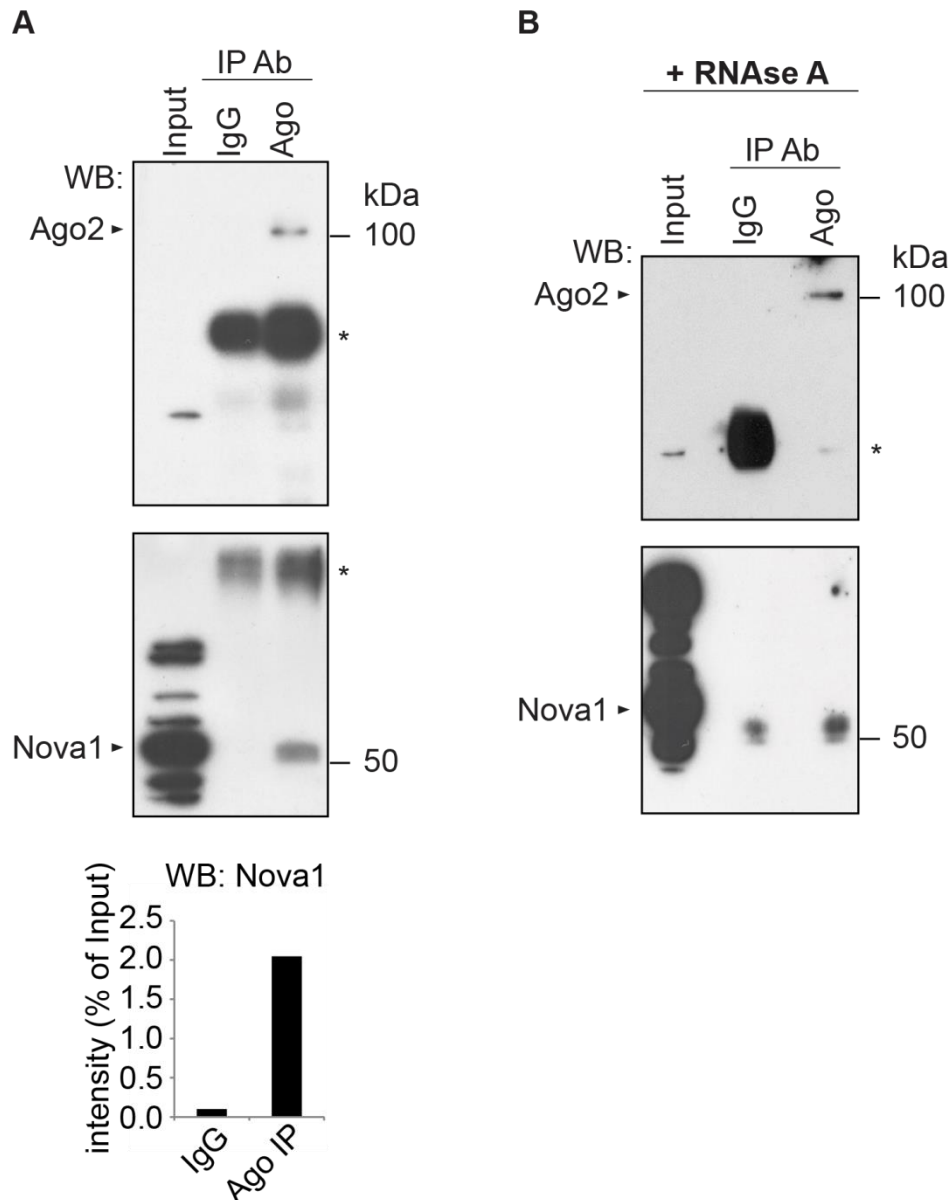


Figure 13: Co-immunoprecipitation of Nova1 and Ago proteins

A) Representative co-IP experiment in hippocampal lysates obtained from adult rat brain using a pan-Ago antibody. Upper part: Western blot using anti-Ago2 antibody exhibits specific precipitation of Ago2 in pan-Ago IP. Lower part: Western blot using anti-Nova1 antibody exhibits specific co-precipitation of Nova1 (52 kDa) and Ago proteins. *, non-identified cross-reactive proteins. Diagram at the bottom represents band intensity as percentage of input averaged over two independent experiments. **B)** Co-IP performed as in A) with additional RNase treatment of lysates before co-IP experiment.

3.5.2 Validation of Nova1 / mRNA association by RNA immunoprecipitation

To further validate a potential interaction between Nova1 and miRISC, I next investigated whether Nova1 is associated with miRNA target mRNAs. Therefore, endogenous Nova1 protein was immunoprecipitated using a Nova1-specific antibody in hippocampal lysates obtained from adult rat brain. Protein and RNA extracts were prepared from these precipitates. The pulldown of Nova1 protein was confirmed by Western blot analysis (Figure 14 A). Nova1 was strongly enriched in the Nova1-IP fraction compared to the IgG control fraction. The presence of specific mRNAs in the Nova1 IP fraction was assessed by quantitative Real Time PCR (qPCR). The bar graph in Figure 14 B represents the relative amount of mRNAs in either IgG or Nova1 IP fraction compared to the input. *Limk1* mRNA was highly enriched in the Nova1 IP compared to IgG control IP. *Rgs4* mRNA was previously shown to interact with Nova1 and served as a positive control (Licatalosi *et al.* 2008). *Gapdh* mRNA, which is not known to be Nova1-associated, was used as a negative control. Together, the results suggest that Nova1 is associated with endogenous miRNA target mRNAs, such as the miR-134 target *Limk1* mRNA. Since Nova1 interacts with Ago proteins, this result is likely due to an indirect association of Nova1 with miRNA targets via miRISC.

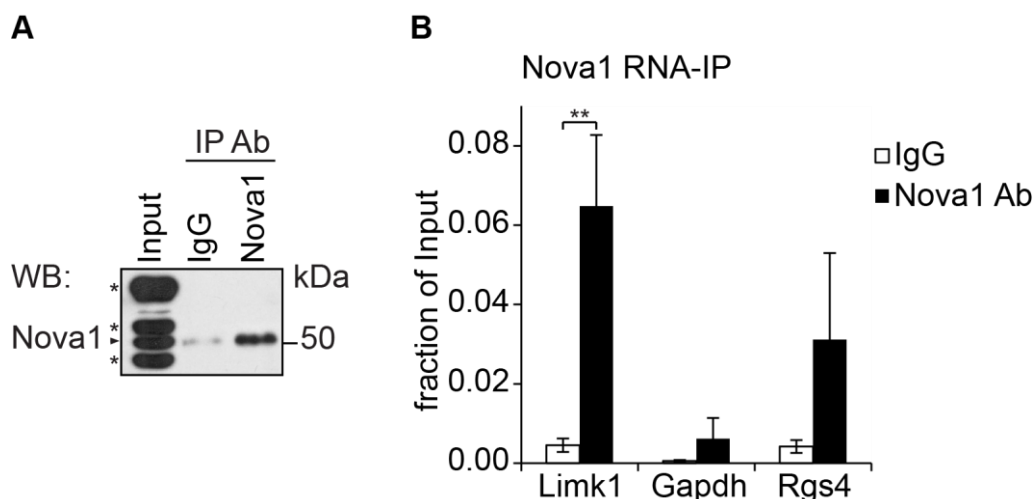


Figure 14: Nova1 RNA-immunoprecipitation in hippocampal lysates

A) RNA-immunoprecipitation (RIP) in rat adult hippocampus using a Nova1 specific or an IgG control antibody. Specific IP of Nova1 was confirmed by Western blot analysis. *, non-identified cross-reactive proteins **B)** qPCR for the indicated transcripts in RIP samples. Bars represent the ratio of mRNA present in Nova1 or IgG control IPs compared to mRNA present in input averaged over three independent experiments \pm standard deviation. **, $p < 0.01$ (unpaired Student's t-test).

3.5.3 Luciferase-based tethering assay of full length Nova1 protein

Having shown that Nova1 associates with miRISC and miRNA target mRNAs, I further investigated the functional activity of Nova1 in the regulation of mRNA translation. I examined whether Nova1 can regulate gene expression independently of its ability to bind RNA by performing a luciferase-based tethering assay (Pillai *et al.* 2005). In this assay the protein of interest is recruited to the 3'UTR of a luciferase reporter through an artificial RNA protein interaction. This allows the experimental separation of protein function from its interaction with RNA. Here, Nova1 is fused to the lambda-phage N-peptide that can directly bind to an RNA hairpin structure (5boxB) that is engineered downstream of the luciferase gene within the reporter vector. First the correct expression of the NHA-fusion constructs was validated by Western blot analysis of lysates obtained from HEK293 cells that were transfected with the respective vectors. A band at the expected size was detected for all constructs (Figure 15 A). Next, I performed luciferase experiments in rat cortical neurons and HEK293 cells using the indicated tethering assay constructs. When NHA-Nova1 was tethered to the reporter construct, the luciferase activity was significantly decreased (0.40 ± 0.08 (CTX); 0.40 ± 0.06 (HEK293)) compared to the NHA-GFP (0.88 ± 0.11) or NHA-Ncoa3 (0.95 ± 0.11) control conditions (Figure 15 B, C). Likewise tethering the miRISC protein Tnrc6c (NHA-Tnrc6c), which serves as a positive control, strongly reduced the reporter activity (0.20 ± 0.10 (CTX); 0.09 ± 0.02 (HEK293)) compared to the control. Expression of the NHA domain alone had no effect on luciferase activity (1.22 ± 0.03) (Figure 15 B). This result shows that Nova1 is capable of repressing mRNA translation independently of its RNA binding capacity. It further indicates that Nova1 possesses an intrinsic repressive activity on mRNA translation, either directly or by recruiting additional translational repressors to the mRNA.

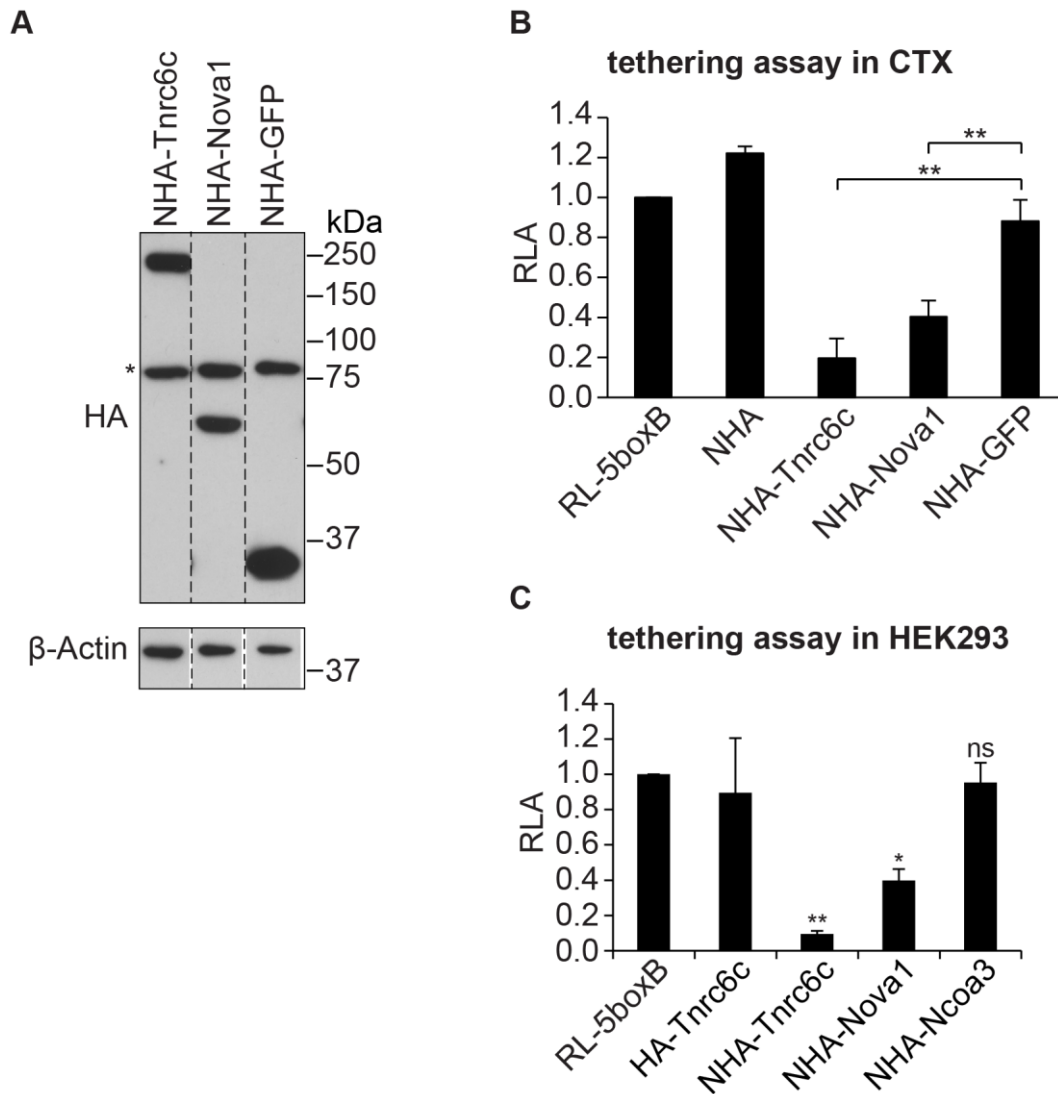


Figure 15: Tethering assay of Nova1

A) Western blot analysis of lysates obtained from HEK293 cells that were transfected with the indicated NHA fusion constructs. Anti-HA antibody was used for detection of fusion proteins and β -actin served as a loading control. *, non-identified cross-reactive proteins **B), C)** Luciferase assay in 7 DIV old rat cortical neurons (**B**) and HEK293 cells (**C**) that had been transfected with the luciferase expression vector (RL-5boxB) together with the indicated fusion constructs. Relative luciferase activity (RLA) represents the ratio of renilla reporter to firefly control reporter normalized to a condition expressing RL-5boxB only. Values are the average from at least three independent experiments \pm standard deviation. ns, not significant *, $p < 0.05$ **, $p < 0.01$ (unpaired Student's t-test compared to RL-5boxB only or as indicated by bars).

3.5.4 Luciferase-based tethering assay of Nova1 deletion constructs

The previous results revealed a repressive function of Nova1 in mRNA translation (Figure 15). To narrow down the region which could facilitate its repressive activity, I generated two deletion mutants of Nova1 and analyzed them in a tethering assay. The two truncated Nova1 fusion proteins consist of either the first 236 amino acids of Nova1 (NHA-Nova1 (1-236)) or of amino acid 237 to 506 (NHA-Nova1 (237-506)). First the subcellular localization of the deletion constructs was studied in cultured hippocampal neurons by immunocytochemistry using an anti-HA antibody. The construct expressing the N-terminal part of Nova1 (NHA-Nova1 (1-236)) lacks the nuclear export signal (NES) and is therefore only present in the nucleus (Figure 16 A). In contrast, the construct expressing the C-terminal part of Nova1 (NHA-Nova1 (237-506)) that lacks the nuclear localization signal (NLS) was only detected in the cytosol (Figure 16 A). The expression of these proteins was further examined by Western blot analysis of lysates obtained from HEK293 cells that were transfected with the two Nova1 deletion constructs. A signal at the estimated size of about 26 kDa was detected with the anti-HA antibody for both constructs (Figure 16 B). Next, the luciferase-based tethering assay was performed in rat cortical neurons using wildtype Nova1 protein and the Nova1 deletion mutants. In contrast to the Nova1 full-length protein (0.46 ± 0.10) none of the mutants were able to repress the luciferase reporter significantly when being tethered to the reporter plasmid (0.96 ± 0.09 (NHA-Nova1(1-236)); 0.88 ± 0.35 (NHA-Nova1 (237-506))) (Figure 16 C). In conclusion, a combination of domains present in both the N- and C-terminus of Nova1 appears to be required for the full repressive activity of Nova1.

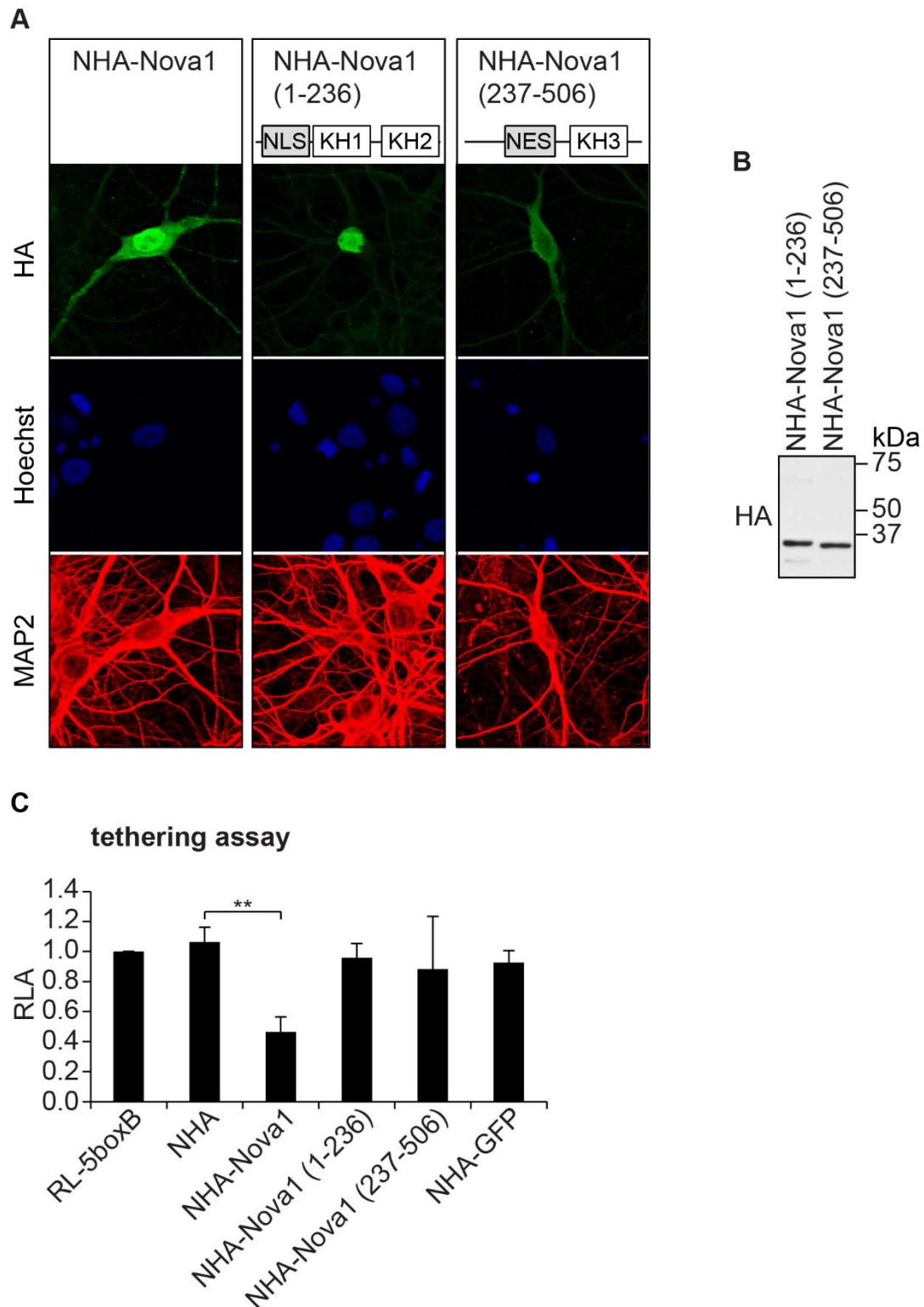


Figure 16: Tethering assay of Nova1 deletion constructs

A) Immunocytochemistry of indicated NHA-Nova1 deletion constructs transfected into cultured hippocampal neurons at 14 DIV. Fusion proteins were detected with an anti-HA antibody (green). Neuronal cells were visualized by Map2 staining (red). Nuclei were stained with Hoechst (blue). **B)** Western blot analysis of lysates obtained from HEK293 cells that were transfected with the indicated NHA fusion constructs. Anti-HA antibody was used for their detection **C)** Luciferase assay in 7 DIV old rat cortical neurons that had been transfected with the luciferase expression vector (RL-5boxB) together with the

indicated fusion constructs. Relative luciferase activity (RLA) represents the ratio of renilla reporter to firefly control reporter normalized to a condition without additional expression vector ("RL-5boxB only"). Values represent averages from three independent experiments \pm standard deviation. **, $p < 0.01$ (unpaired Student's t-test).

3.6 The function of Nova1 in neuronal morphogenesis and signal transduction

3.6.1 Nova1 is required for miR-134 mediated spine morphogenesis

MiR-134 regulates dendritic spine size by repressing the translation of Limk1 mRNA, which encodes an actin regulatory protein kinase that promotes spine growth. It was shown that the spine size of hippocampal neurons was significantly reduced upon overexpression of miR-134 (Schratt *et al.* 2006). In previous experiments of this thesis it was observed that Nova1 is interfering with the miR-134 mediated repression of the Limk1 expression in luciferase reporter gene assays and that Nova1 furthermore associates with Limk1 mRNA. To test whether Nova1 function is required for miR-134 mediated spine size reduction a spine assay was performed in mature hippocampal neurons. The spine assay was performed in collaboration with Peter Störchel (Störchel and Thümmel *et al.* 2015). A Nova1 shRNA expressing pSuper vector was co-transfected with GFP into cultured hippocampal neurons at 13 DIV in the presence or absence of miR-134 duplex RNA. The cells were fixed after six days and the spines were visualized by confocal microscopy followed by the quantitative measurement of spine volume based on GFP filling (chapter 2.7). In order to reduce variability, only spines along the basal dendrites were considered. Overexpression of miR-134 led to a significant reduction of the spine size in hippocampal neurons (Figure 17). This result is in accordance with the reported findings in Schratt *et al.* in 2006. The miR-134 induced spine size reduction was abolished after Nova1 knockdown by shRNA. However, spine size was reduced in a condition with an empty vector control. This shows that Nova1 is required for the miR-134 mediated spine size reduction in hippocampal neurons and that Nova1 regulation of miRNA activity is functionally relevant for the morphology of synapses.

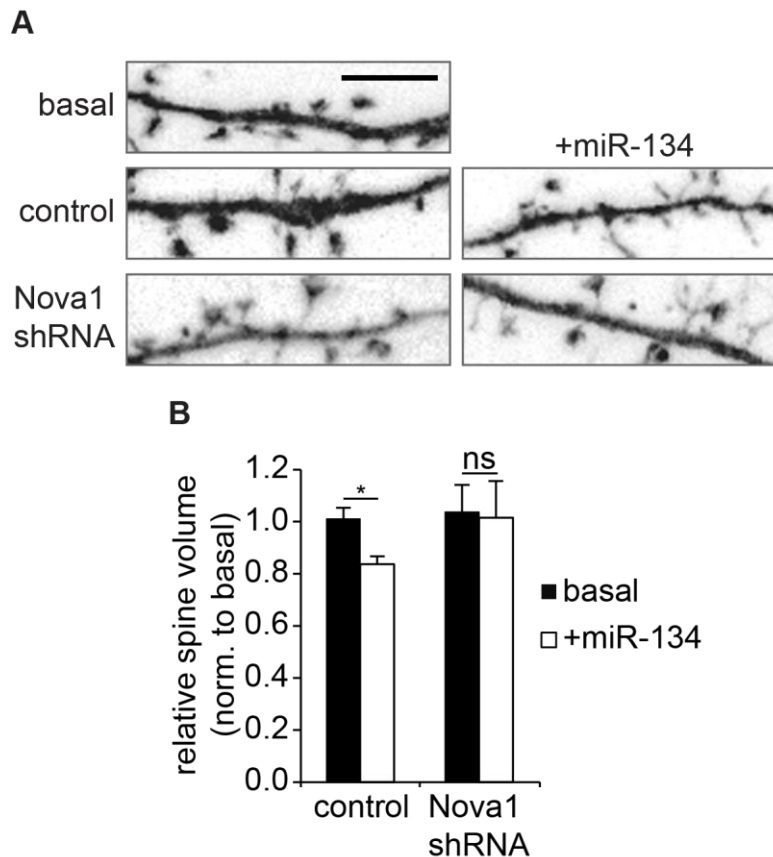


Figure 17: Spine assay in hippocampal neurons upon Nova1 knockdown and miR-134 overexpression

A) Spine analysis of hippocampal neurons (19 DIV) that were transfected at 13 DIV with Nova1 shRNA expressing pSuper vector or an empty control vector together with pEGFP in the presence or absence of miR-134 duplex RNA (+miR-134). Shown are representative sections of dendrites with dendritic spines. Scale bar = 5 μ m **B)** Bars represent quantified relative spine volume normalized to a basal condition without shRNA expression vector. Values represent averages from three independent experiments each including 6 cells per condition \pm standard deviation. ns, not significant *, $p < 0.05$ (unpaired Student's t-test). The spine assay was carried out by Anna Tsankova under supervision of Peter Störchel.

3.6.2 The role of Nova1 in dendrite outgrowth

It was previously shown from our group that besides controlling neuronal spine growth miR-134 is involved in the activity-dependent regulation of dendrite outgrowth. In this context, miR-134 inhibits the translation of the translational repressor Pumilio2 (Pum2) that results in an increase of dendritic outgrowth of hippocampal neurons (Fiore *et al.* 2009). To test whether Nova1 plays a role in the regulation of dendritogenesis in hippocampal neurons, dendritic complexity was assessed upon Nova1 knockdown under basal conditions. The analysis of dendritic outgrowth was performed in collaboration with Gabriele Siegel (Störchel and Thümmel *et al.* 2015). Cultured hippocampal neurons

were transfected with a Nova1 shRNA expression vector together with pEGFP and prepared for confocal microscopy at DIV10. The dendritic outgrowth of the cells was determined using the Sholl method. Microscopic images of single neurons were superimposed with concentric circles of different radii around the cell body. The intersections of dendrites with the circles were quantified at each distance. The dendritic complexity was not affected under Nova1 knockdown conditions in areas close to the cell body (25-100 μm) compared to the shRNA control (Figure 18 A). In contrast, at circles with more distance to the cell body (125-175 μm) the dendritic growth was significantly reduced upon Nova1 knockdown. However, the summation of all intersections with every circle showed no significant difference of dendritic outgrowth in the Nova1 knockdown compared to the control shRNA (Figure 18 B). This suggests that Nova1 has no major functional relevance in the regulation of dendritic outgrowth under basal conditions. However, Nova1 is specifically required for the proper development of dendrites at large distances from the neuronal soma, possibly via regulation of dendritic miRNA activity.

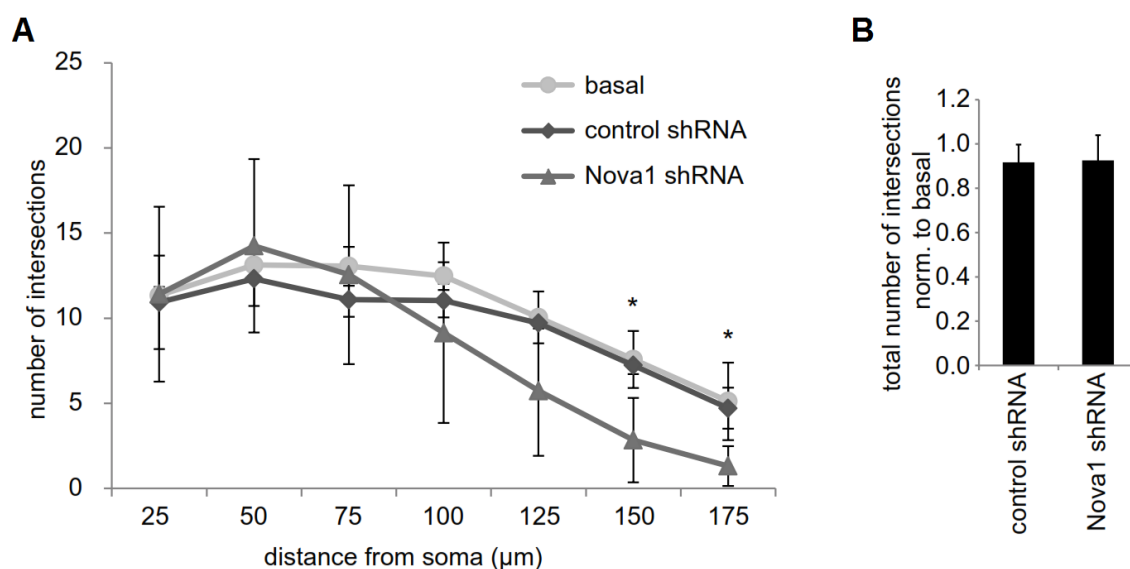


Figure 18: Dendritic outgrowth after Nova1 knockdown

A) Dendritic outgrowth of cultured hippocampal neurons (10 DIV) that were transfected at 4 DIV with Nova1 shRNA expressing pSuper vector or an empty control vector together with pEGFP was determined by Sholl analysis. **B)** Bars represent the accumulated number of intersections from data in A and normalized to a basal condition without shRNA expression vector. Values represent averages from three independent experiments \pm standard deviation. *, $p < 0.05$ (unpaired Student's t-test). Data was obtained by Gabriele Siegel.

3.6.3 The function of Nova1 in BDNF signaling

I found that Nova1 is required for miR-134 mediated spine shrinkage in hippocampal neurons (chapter 3.6.1). It was previously shown that the inhibitory effect of miR-134 on spine growth can be reversed by the application of brain-derived neurotrophic factor (BDNF) (Schratt *et al.* 2006). This prompted me to analyze the function of Nova1 in BDNF-regulated Limk1 mRNA translation using a Limk1 3'UTR luciferase reporter vector containing a destabilized firefly open reading frame (LIMK1-lucPEST). The luciferase protein encoded by this vector has a higher turnover rate than the conventional luciferase protein. Therefore, this system can better monitor stimulus-dependent alterations in gene expression compared to the conventional, very stable luciferase reporter system. Under normal growth conditions, Nova1 knockdown caused an about 2-fold increase of the luciferase expression (2.16 ± 0.64) compared to the control shRNA condition (1.08 ± 0.42) (Figure 19 A). 5 h BDNF treatment of neurons induced a significant upregulation of the reporter expression in the condition without shRNA expression construct ("no shRNA") (1.00 (basal); 2.37 ± 0.41 (BDNF)). Under Nova1 knockdown this BDNF-mediated increase was impaired (2.16 ± 0.64 (basal); 2.65 ± 0.36 (BDNF)) compared to control shRNA condition (1.08 ± 0.42 (basal); 2.03 ± 0.35 (BDNF)), suggesting that Nova1 is required for the BDNF mediated upregulation of the Limk1 reporter (Figure 19 A). Both BDNF application and Nova1-knockdown induced an upregulation of the Limk1-lucPEST which raises the possibility that Nova1 is a downstream effector of BDNF signaling. For this reason, Nova1 protein levels were examined in cortical neurons after BDNF treatment for different durations. The Western blot analysis for Nova1 after incubation with BDNF for different timepoints between 5 min to 5 h however did not reveal significant changes of total Nova1 protein levels (Figure 19 B). Taken together, these results suggest that Nova1 is involved in the dynamic control of Limk1 mRNA translation in response to BDNF signaling. Since BDNF did not affect Nova1 protein levels, the inactivation of Nova1 by BDNF might be caused by post-translational modifications.

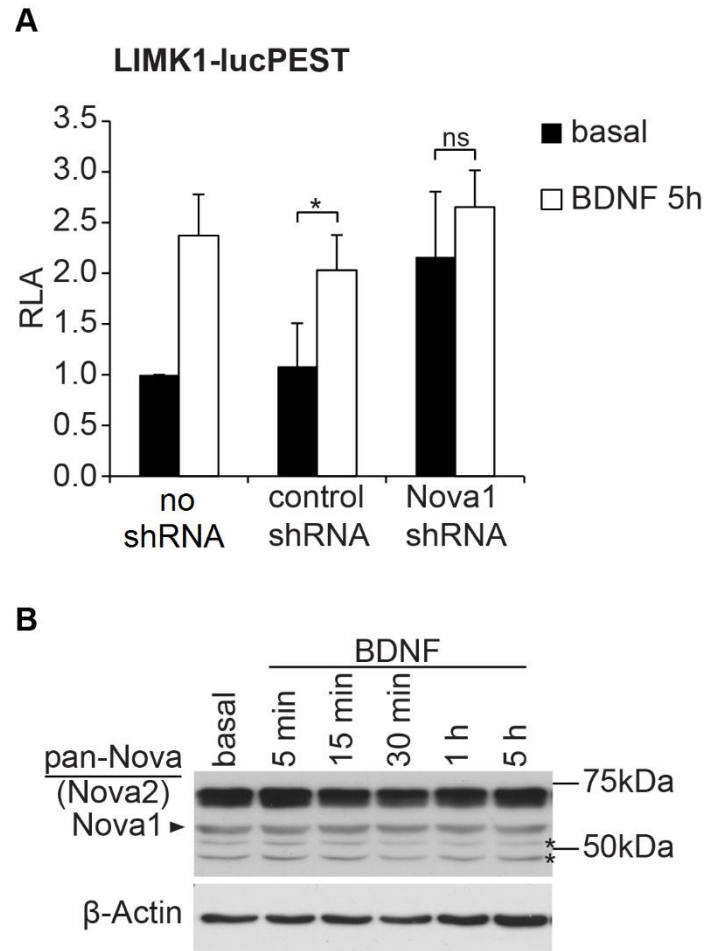


Figure 19: Luciferase assay and Western blot analysis upon BDNF treatment

A) Luciferase assay in rat cortical neurons (7 DIV) that had been transfected at 5 DIV with a Limk1 3'UTR luciferase construct that contains a destabilized firefly ORF (lucPEST) together with pSuper vectors expressing either Nova1 shRNA or control shRNA. Prior luciferase measurement neurons were treated with BDNF (100 ng/ml) or kept untreated ("basal") for 5 h. Relative luciferase activity (RLA) represents the ratio of firefly reporter to renilla control reporter normalized to a condition without shRNA expressing vector ("no shRNA") and no BDNF stimulation. Values represent averages from at least three independent experiments \pm standard deviation. ns, not significant *, $p < 0.05$ (unpaired Student's t-test). **B)** Western blot analysis of lysates obtained from cortical neurons at 14 DIV treated with BDNF for indicated time or mock-treated (basal). Anti-pan Nova antibody was used for detection of Nova1. β -actin served as a loading control. *, non-identified cross-reactive proteins.

4 Discussion

MiRNAs are important regulators of mRNA translation in the nervous system and control important aspects of brain development, neuronal differentiation and synaptic plasticity (Aksoy-Aksel *et al.* 2014; Bicker *et al.* 2014). Previous studies showed that specific miRNAs, such as miR134, regulate the local translation of mRNAs at the synapse thereby controlling activity-dependent changes in synaptic strength (Schratt *et al.* 2006). The miRNA-induced silencing complex (miRISC) plays a crucial role in the miRNA-mediated repression of mRNA translation (Fabian *et al.* 2012). For non-neuronal cells it is known that the activity of miRNAs is modulated by RNA-binding proteins (RBPs) that are not core components of miRISC (Krol *et al.* 2010b). However, miRNA modulating functions of RBPs in neurons are poorly understood. Based on a large-scale RNAi screen, I identified Nova1 as a novel neuron-specific modulator of miRNA function, with potential implications for the regulation of dendritic protein synthesis and synaptic plasticity.

4.1 RNAi based screen in neurons

In this project I validated an initial screening approach from our lab that represents the first reported large scale functional RNAi screen in primary neurons (Störchel and Thümmel *et al.* 2015). Among the positive hits of the screen were the two known miRISC factors Tnrc6c and Ddx6, which demonstrates that the screen setup enables the identification of established factors in miRNA function. Tnrc6c and Ddx6 are direct Ago-interacting proteins that play essential roles in miRNA-mediated gene silencing (Chu *et al.* 2006; Pfaff *et al.* 2013). Importantly, three new factors (Nova1, Ncoa3 and Ewsr1) that are required for miRNA function emerged from the whole screening study (Figure 6). So far, these proteins were neither associated with miRNA function nor found in other published studies that described miRNA regulatory proteins in non-neuronal cell systems (Eulalio *et al.* 2008). This suggests that neuronal miRNA activity is subject to regulation by a specific subset of RNA-binding proteins.

The knockdown of the other RBPs that were classified as positive candidates in the large-scale screen did not reveal any significant relief of reporter repression in a more specific

secondary screen. These proteins therefore might play a more general role in the regulation of mRNA translation not directly related to miRNA-dependent repression.

4.2 Nova1 is expressed in the nucleus and cytoplasm of neurons

Nova proteins (Nova1 and Nova2) were first characterized as onconeural antigens in the human motor disorder paraneoplastic opsoclonus myoclonus ataxia (POMA) that occurs in breast or lung cancer patients (Buckanovich *et al.* 1993). Nova1 and Nova2 are related proteins that have 75% sequence homology and are particularly conserved within their KH domains (Lewis *et al.* 1999). Both Nova genes were among the 286 RBPs that were analyzed in the initial screen. However, in contrast to Nova1, Nova2 knockdown did not significantly alter miR-134 mediated reporter repression and for this reason was not considered for follow-up experiments (Siegel, 2011). This finding indicates specific, non-redundant functions of Nova proteins in post-transcriptional gene regulation.

Nova1 was shown to regulate alternative splicing in neurons and to target a set of mRNA transcripts that encode components of inhibitory synapses (Jensen *et al.* 2000; Ule *et al.* 2003). The expression of Nova1, which is restricted to subcortical parts of the central nervous system, was shown to be largely confined to the nuclear compartment (Buckanovich *et al.* 1996; Buckanovich *et al.* 1997). However, more recently significant amounts of Nova1 protein were detected in the cytoplasm of mouse brain cells. It was reported that 50% of cellular Nova1 protein localizes to the soma-dendritic compartment. Consistent with this localization pattern, Nova1 contains both a nuclear export (NES) and nuclear localization signal (NLS) that allow the protein to shuttle between the nucleus and cytoplasm (Racca *et al.* 2010).

The results obtained within this thesis further support nucleo-cytoplasmic shuttling of Nova1. First, the results from Western Blot analysis show that Nova1 protein is endogenously expressed throughout the *in vitro* development of hippocampal and cortical neurons between 7 and 14 DIV (Figure 7 A, B). Furthermore, the protein was detected by immunocytochemistry in the nucleus and in the cytoplasm of hippocampal neurons. The majority of cytoplasmic Nova1 was observed in a granular pattern surrounding the nucleus (Figure 7 C, D). Comparing the relative amount of protein in both cell compartments

by Western blotting, I observed a prominent expression of Nova1 in the nucleus (Figure 7 E).

Similarly, the study from Racca *et al.* detected the majority (~60%) of Nova1 protein in the nucleus compared to the cytoplasm when it was normalized to total protein amount which was done in my experiment as well. On the one hand, this result is in agreement with the function of Nova1 as a nuclear splicing factor. On the other hand, these observations suggest an alternative cytosolic function of Nova1. In fact, the same study reported that Nova co-localizes with its target mRNAs (e.g. GlyR α 2) within dendrites of spinal cord motor neurons. Likewise, an earlier study reported a granular localization of Nova1 in the cytoplasm and in neurites of motoneurons in a manner overlapping with nELAV, another neuronal RBP (Ratti *et al.* 2008). The final model of Racca *et al.* suggests a combined function of Nova that comprises the regulation of alternative splicing in the nucleus and the regulation of mRNA expression in the cytoplasm (Racca *et al.* 2010). In agreement with this hypothesis earlier studies already provided indirect evidence that Nova1 protein might have a regulatory function in the cytoplasm. For example, HITS-CLIP analysis of Nova proteins (Nova1 and Nova2) in mouse brain revealed interactions of Nova with 3' untranslated regions of RNAs and further discovered that Nova regulates alternative polyadenylation of mRNAs in the brain (Licatalosi *et al.* 2008).

In conclusion, the findings of my thesis together with previous studies strongly suggest that Nova1 acts as a regulator of post-transcriptional gene expression in the cytoplasm.

4.3 Nova1 is required for miRNA function

Nova1 was shown to associate with the 3'UTR of a huge number of mRNAs (Licatalosi *et al.* 2008) which led to the hypothesis that Nova1 regulation is not limited to miR-134 but affects other miRNAs, as well. Another functionally important miRNA in the brain is miR-138. It mediates the translational repression of the depalmitoylation enzyme APT1 and thereby regulates the size of dendritic spines (Siegel *et al.* 2009).

Results from luciferase assays that were performed in the context of overexpressed as well as endogenously expressed miR-138 revealed that Nova1 is also required for the activity of miR-138 (Figure 8 and Figure 9). This suggests that Nova1 does not act

exclusively miR-134 - specific but might play a more general role in the regulation of miRNA activity.

Further luciferase based experiments provided evidence that the observed effects of Nova1 knockdown on the miRNA-mediated mRNA repression were indeed dependent on the activity of miRNAs. By analyzing different miRNA targets (LIMK1, IQGAP1, LIN41 and HMGA2) in luciferase reporter assays using mutated miRNA binding sites, I found that Nova1 regulates most of the tested target mRNAs in a miRNA-dependent manner (Figure 10). One exception is the HMGA2 reporter that was shown to be regulated by Nova1 in a let-7-independent manner. This observation could be explained by the existence of other miRNA binding sites in the HMGA2 3'UTR that are regulated by Nova1. Since my data suggests Nova1 as a general miRNA regulator, other miRNAs targeting HMGA2 might cover a let-7-dependent effect. This theory is supported by the finding that most of the miRNA binding site mutations do not completely abolish the Nova1-mediated increase of the reporter expression. However, this data is also consistent with a miRNA-independent component in Nova1 regulated mRNA translation.

With respect to the observation that Nova1 can act in a miRNA-dependent manner, it was important to test whether the availability of a miRNA binding site alone is sufficient for the regulatory function of Nova1. Results from a luciferase assay with a reporter lacking 3'UTR sequences flanking miRNA binding sites revealed that the miRNA binding sites alone are sufficient to mediate the effect of Nova1 knockdown (Figure 11). This suggests that Nova1 can act as a regulator of miRNA activity independent of the 3'UTR sequence.

4.4 Nova1 associates with miRISC and mRNA

The miRNA-mediated translational repression of mRNA targets requires the association of AGO protein with a GW182 (Tnrc6) protein. The Ago-GW182 interaction forms the core of the miRISC, a multiprotein complex that is recruited by miRNAs to their respective target mRNAs (Fabian *et al.* 2012). The correct function of the miRISC is controlled by associated proteins that interact directly or indirectly with either the core miRISC or the target mRNA. For example, some of the miRISC interacting proteins belong to the family of DEAD-box RNA helicases like Ddx6 and Mov10, which interact with Ago proteins to facilitate miRNA-mediated translational repression (Chu *et al.* 2006;

Nicklas *et al.* 2015; Banerjee *et al.* 2009). Other modulators of miRNA activity, like Fmrp and Rbm4 directly or indirectly associate with AGO but also interact with AGO-associated miRNAs and target mRNAs (Edbauer *et al.* 2010; Li *et al.* 2014; Lin *et al.* 2009). Interestingly, Rbm4 and Smd1 are required for miRNA activity, independent of their essential function in mRNA processing (Xiong *et al.* 2015). Similarly, we envision that Nova1 might facilitate miRISC function in neurons independently of its originally described role in the regulation of alternative splicing.

Since many of the miRISC modulating proteins are associating with Ago proteins, I hypothesized that Nova1 might regulate miRNA function via an interaction with Ago. I showed using co-Immunoprecipitation (co-IP) experiments with rat hippocampal lysates that Ago protein was precipitated with a pan-Ago antibody that can recognize all four Ago proteins. The successful pulldown was confirmed for Ago2 (Figure 13). Since a positive precipitation of Ago1, Ago3 and Ago4 was not tested, the assay only allows a conclusion about a potential interaction between Nova1 and Ago2 protein. Indeed, I observed a specific co-precipitation of Nova1 in the Ago-IP fraction which indicates that Nova1 interacts with Ago2 (Figure 13 A). Further co-IP experiments revealed that this interaction does not require the presence of RNA which suggests that Nova1 and Ago associate in a complex via protein-protein interaction (Figure 13 B).

Despite the fact that Nova1 and Ago were successfully co-immunoprecipitated, some limitations of the co-IP experiment should be considered for future studies. First, due to the reported association of Ago with cellular membranes (Cikaluk *et al.* 1999; Stalder *et al.* 2013), Ago IP required harsh lysis conditions that in principle could impair a Nova1-Ago interaction. This could explain the relatively small amounts of Nova1 that were detected in the Ago-IP. I further observed that in other cell systems, like cultured primary cortical neurons, the Nova1-Ago interaction was not preserved with the same buffer conditions. To overcome this problem in future experiments it might be helpful to use milder lysis conditions that preserve protein-protein interactions better. Regarding the interaction of Nova1 with Ago, another approach for future analysis could be an *in vitro* binding assay of Ago and Nova1 that includes Nova1 constructs with mutations in potential binding sites for Ago. Another limitation of the experiments was the inability to detect Ago proteins in Nova1-IPs. A possible reason could be that the Nova1 antibody masks the binding site for Ago which would perturb the Nova1-Ago interaction.

However, this interpretation is unlikely, since I used several different Nova1 antibodies, most of which efficiently worked in IP experiments (data not shown).

All together my data argues for a relatively weak interaction of Nova1 with Ago but suggests that Nova1 regulates the expression of miRNA target mRNAs through an association with miRISC. Moreover, it is likely that additional protein binding partners of Nova1, like translation factors, might be required for its potential function in translational repression.

A potential association of Nova1 with the miRISC was further supported by my analysis of interaction between Nova1 and miRNA target mRNAs. RNA-immunoprecipitation (RIP) experiments revealed that Nova1 specifically interacts with the endogenous miR-134 target mRNA *Limk1* (Figure 14). In principle, this result could support a model whereby Nova1 directly contacts miRNA target mRNAs, such as *Limk1*. However, this hypothesis is not in agreement with the results from the co-IP that showed an RNA-independent interaction of Nova1 with miRISC. Therefore, it is more conceivable that Nova1 indirectly associates with mRNA via components of the miRISC (e.g. Ago), since the buffer conditions used of the RIP are expected to preserve protein-protein interactions.

The Nova1 association with *Limk1* mRNA is in accordance with the observation that Nova1 represses the expression of the *Limk1*-luc reporter in a miR-134-dependent manner (Figure 10 A). Moreover, results from Western Blot analysis of lysates from Nova1 depleted cells support the repressive function of Nova1 on *Limk1* expression. Endogenous protein amount of *Limk1* was significantly increased upon knockdown of Nova1 in hippocampal lysates (Figure 12).

Furthermore, the artificial recruitment of Nova1 to the 3'UTR in the context of an mRNA tethering assay showed that Nova1 is able to induce translational repression independent of its RNA binding activity (Figure 15). Considering the fact that Nova1 can regulate miRNA activity independently of the 3'UTR context (Figure 11), we envision that Nova1 might act towards miRISC activity in a more general way that does not necessarily involve specific RNA contact. This also suggests that Nova1 would regulate the translation of a vast majority of miRNA targets that are expressed in neurons. However, Nova1 might still have some target specificity due to the existence of different pools of miRISCs which either contain Nova1 or not. Putative determinants could be the miRNA

sequence, the complementarity of the miRNA at the binding site, the identity of the Ago protein or other associated unknown factors.

Nova1 is well known for the ability to bind RNA by virtue of its three K-homology (KH) type domains (Buckanovich *et al.* 1997). These three KH domains are connected through small linker elements and span the major part of the protein sequence beside two motifs for nuclear localization and export. Although the amino acid structure of Nova1 does not contain motifs that are known to mediate protein-protein interactions, several Nova1 interaction partners have been described in the literature.

First, yeast-two hybrid identified the brain-enriched polypyrimidine tract-binding protein (brPTB) as a Nova interacting factor (Polydorides *et al.* 2000). Interestingly, brPTB is closely related to the polypyrimidine tract-binding protein (PTB) that was later implicated in the regulation of miRNA function (Engels *et al.* 2012; Xue *et al.* 2013). Furthermore, Nova1 shares sequence and functional similarities with the known miRISC associated factor FMRP (Jin *et al.* 2004b; Buckanovich *et al.* 1996). FMRP is associated with the miRNA processing machinery and interacts with Ago (Jin *et al.* 2004b; Edbauer *et al.* 2010; Caudy *et al.* 2002). By comparing Nova1 and FMRP it is striking that both contain a KH1 and KH2 domain as well as NLS and NES motifs (Valverde *et al.* 2007). Furthermore, a crystallization study about binding properties of Nova1 KH1 and KH2 domains reported that KH2 might be involved in protein-protein contacts (Teplova *et al.* 2011).

Results from tethering assays with two truncated variants of the Nova1 protein provided more insight into the parts of Nova1 that could facilitate its repressive activity. In contrast to the Nova1 full-length protein, none of the truncated proteins were able to repress the luciferase reporter significantly when being tethered to the reporter plasmid (Figure 16 C). This suggests that a combination of domains present in both the N- and C-terminus of Nova1 is required for the repressive function of Nova1. Nevertheless, to uncover a specific domain or protein region that mediates this function, a more detailed domain mapping will have to be performed in future studies.

To further reveal the mechanism that underlies Nova1 function in miRNA activity, it is important to investigate new direct binding partners of Nova1. A recent study used yeast two hybrid screens of mouse cDNA libraries with full-length Nova bait constructs in order to identify Nova-interacting proteins (Polydorides *et al.* 2000). However, this approach

does not provide the neuronal context that might be required for the Nova1 repressive function. This could be achieved for example by immunoprecipitation of Nova1 in lysates of neuronal origin followed by mass spectrometry to identify co-precipitated proteins.

4.5 The significance of Nova1 for neuronal morphogenesis and function

The functional relevance of miR-134 mediated mRNA inhibition includes the regulation of spine morphology and dendritic outgrowth. In particular, dendritic spine size is regulated by miR-134 through the repression of *Limk1* mRNA (Schratt *et al.* 2006). *Limk1* promotes actin polymerization and spine growth by phosphorylating ADF/cofilin proteins (Bamburg. 1999). The inhibition of *Limk1* by miR-134 in turn promotes spine shrinkage. I found that Nova1 is necessary for the miR-134-mediated repression of the *Limk1*-luc reporter and additionally interacts with the *Limk1* mRNA (Figure 10, Figure 14). Importantly, I could further show that Nova1 deficiency, although not altering spine development on its own, specifically interfered with the miR-134-induced shrinkage of spines (Figure 17). In conclusion, this suggests that Nova1 does play a role in neuronal function, specifically in the miR-134-mediated spine size reduction. However, considering that Nova1 was also shown to be required for the activity of other neuronal miRNAs like miR-138 and miR-124, it is tempting to speculate that Nova1 function might be also relevant in the context of other miRNAs with reported neuronal functions. For example, overexpression of miR-138 induces shrinkage of spine volume, similar to miR-134 (Siegel *et al.* 2009). Therefore, one approach for future studies would be to investigate whether Nova1 is required for the miR-138 mediated spine size reduction by analyzing spine morphology upon Nova1 knockdown in the context of miR-138 overexpression.

BDNF is a neurotrophin that is synthesized and released upon stimulation of neuronal activity. It was shown that BDNF can release the inhibitory effect of miR-134 on *Limk1* mRNA translation (Schratt *et al.* 2006). However, the molecular mechanisms that are responsible for the BDNF-dependent inhibition of miRNA repressive activity are unknown.

Since I found that the neuron-specific Nova1 protein is associated with miRISC, I further tested an involvement of Nova1 in the BDNF-mediated regulation of miR-134 activity.

First, results from luciferase assays showed that the treatment of BDNF induces a significant upregulation of the Limk1-luc reporter expression (Figure 19 A). This supports the previous findings reported in Schratt *et al.* (2006) that demonstrated a significant induction of translation of a Limk1-3'UTR luciferase reporter upon BDNF treatment. Moreover, results from luciferase assays revealed that under Nova1 knockdown the BDNF-mediated increase of Limk1-luc expression was impaired (Figure 19 A). This suggests that Nova1 might be involved in the dynamic control of Limk1 mRNA translation in the response to BDNF stimulation. However, the molecular mechanism underlying this regulatory function still remains unclear. One possible explanation would be a BDNF-dependent downregulation of Nova1 expression. However, I could not obtain experimental support for this hypothesis in my thesis. Results from Western blot analysis of lysates from BDNF-treated cortical neurons did not detect any changes in the total amount of Nova1 protein compared to untreated cells. (Figure 19 B). Therefore, BDNF might not affect total protein amounts of Nova1 but could control its subcellular localization and/or repressive activity by inducing specific post-translational modifications. Interestingly, Nova1 was shown to harbor serine and threonine rich domains that serve as potential phosphorylation sites. A serine residue located in exon E4 was identified as a major phosphorylation site (Dredge *et al.* 2005). However, its functional relevance is still unclear. It is conceivable that BDNF could lead to alterations in the phosphorylation state of Nova1, thereby modulating its repressive function in the context of miRNA-dependent Limk1 mRNA translation.

To test these hypotheses in the future, a more detailed analysis of the Nova1 subcellular localization in neurons is required, e.g. by performing Nova1 Western blot with nuclear and cytoplasmic fractions of BDNF treated neurons. Another approach would be to analyze the phosphorylation state of Nova1 upon BDNF stimulation with a phospho-specific Nova1 antibody, which would require the previous identification of the exact phosphorylation site, e.g. using mass spectrometry.

It is tempting to speculate that Nova1 function is specifically required for dynamic activity-dependent changes in neuronal morphology, such as those occurring in synaptic plasticity. In this regard, it would be interesting to investigate whether the interaction between Ago and Nova1 is modified by synaptic stimuli or related growth factors (e.g. BDNF).

MiR-134 is not only involved in spine morphology but also essential for the activity-dependent regulation of dendritogenesis. This effect is mediated by miR-134-dependent inhibition of the translational repressor Pumilio2 (Pum2). Previous work demonstrated that upon stimulation of hippocampal neurons with KCL or BDNF the dendrite outgrowth was increased in number of branches and total length (Fiore *et al.* 2009).

In order to test whether Nova1 is involved in the regulation of dendritogenesis, I initially investigated the dendritic complexity upon Nova1 knockdown in non-stimulated neurons. The results revealed that Nova1 does not seem to have a major functional relevance in the regulation of dendritic outgrowth under basal conditions (Figure 18 B). An explanation for this observation could be that Nova1 depletion perturbs the activity of most miRNAs (see above). Since neuronal miRNAs were shown to have both inhibitory and stimulatory activity in dendritogenesis, the net effect would be expected to be small. We found, that dendritogenesis in neurons lacking the essential miRNA biogenesis component Droscha was similarly unaltered. The resulting loss of the majority of miRNAs did not cause any significant changes in dendritic outgrowth presumably due to a balance of opposing effects in dendritogenesis (Störchel and Thümmel *et al.* 2015).

Although Nova1 knockdown did not affect overall dendritic complexity, it caused a slight but specific reduction in the growth of distal dendrites (Figure 18 A). This suggests an involvement of Nova1 in the local regulation of distal dendrites. It was shown that dendritic compartmentalization is, among others, modulated by AMPAR-type glutamate receptors (AMPA) whose density increases with increasing distance from the cell soma. AMPAR function and trafficking into synaptic membranes is mediated by specific regulatory proteins (TARPs, transmembrane AMPA receptor regulatory proteins) that were for example shown to promote dendritic growth in either proximal or distal zones of dendrites in pyramidal neurons depending on the age of the cells (Hamad *et al.* 2014). It was further reported that a higher number of AMPA-receptors in the distal dendritic zones is accompanied by higher synaptic transmission. Synaptic properties that are determined by their position in the dendritic tree are known as distance-dependent scaling. This phenomenon is necessary for balancing the weakening of action potential currents that are filtered while traveling along the dendrite (Ito *et al.* 2009; Shipman *et al.* 2013). One possible approach to test whether Nova1 is involved in dendrite compartmentalization would be to investigate the expression or function of AMPAR regulating proteins, such as TARPs, in Nova1-deficient hippocampal neurons.

On the other hand, considering my observation that Nova1 was interfering with the BDNF-induced upregulation of the *Limk1-luc* reporter expression, it might be possible that Nova1 has a more prominent role in activity-dependent dendritogenesis. Future analysis of Nova1 deficiency in dendritic complexity upon neuronal stimulation with BDNF or other activity-related stimuli could yield more information about a putative involvement of Nova1 in this context.

4.6 Conclusion and Outlook

The main aim of this project was to obtain more insight into the molecular mechanism by which miRISC activity is regulated in neurons. Based on a previous large-scale RNAi-based screening study, the RBP Nova1 was identified and further characterized as a new miRISC activator in primary neurons. Originally described as an essential regulator of alternative splicing in the nucleus, Nova1 might therefore also function as post-transcriptional regulatory RBP in the cytoplasm. My findings therefore provide further evidence that RBPs dynamically participate in the post-transcriptional control of gene expression in different cellular compartments.

The main findings related to Nova1 function gathered during this thesis can be summarized in a simple working model (Figure 20). Under basal conditions Nova1 shuttles to the cytoplasm where it can associate with miRISC potentially via a direct protein interaction with Ago proteins. However, which of the 4 mammalian Ago proteins are direct binding partners of Nova1 remains to be investigated. The Nova1-Ago interaction serves to place the Nova1 repressive activity to the translation machinery. How Nova1 interferes with mRNA translation, and whether this is strictly Ago-dependent is still unclear. Therefore, it is very important to elucidate additional Nova1 interacting proteins, e.g. factors associated with translational regulatory complexes, in future studies. Furthermore, Nova1 probably participates in the BDNF-dependent release of miRNA repressive activity, a mechanism which is still highly elusive. How Nova1 function is modulated by BDNF remains an important subject for future experiments.

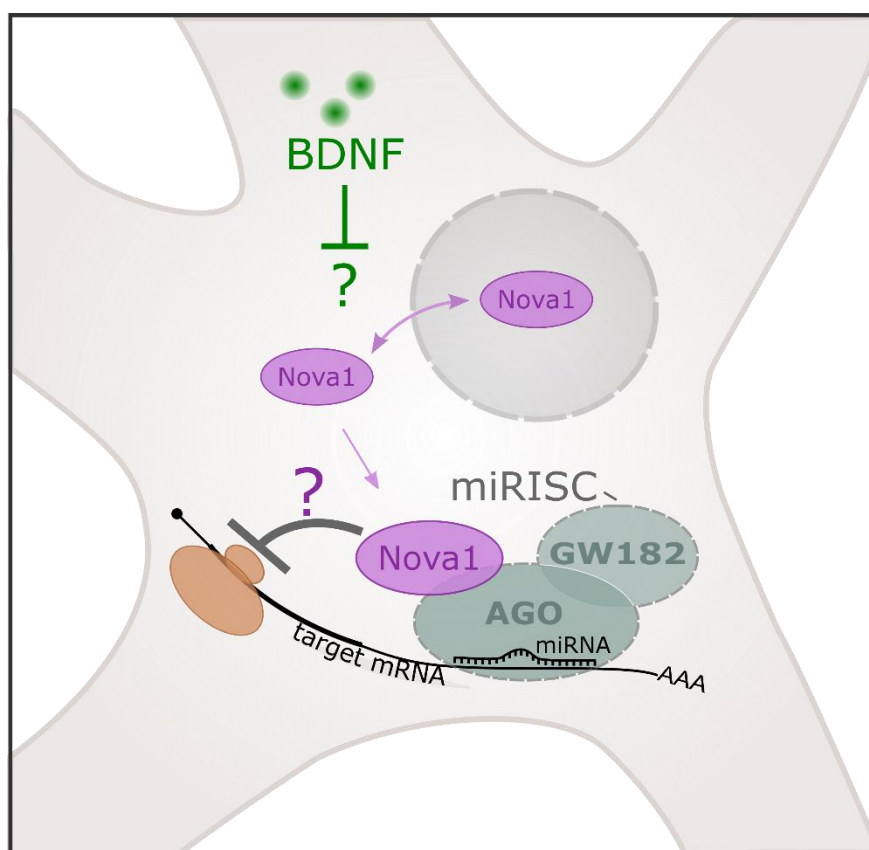


Figure 20: Model of Nova1 function in the regulation of miRNA activity

Nova1 can associate with miRISC in the cytoplasm via a direct protein interaction with Ago. Nova1 promotes translational repression of the Ago loaded miRNA target mRNAs. The Ago-Nova1 interaction might be modified upon BDNF release and thereby contributes to dynamic activity-dependent regulation of miRNA activity.

The loss of Nova1 function as a regulator of miRNA activity in neurons might impair the proper regulation of activity driven processes that control synaptic strength and plasticity. This could have pathophysiological consequences for the human nervous system like for example in schizophrenia and epilepsy. These diseases were reported to be associated with alterations in specific miRNAs, including miR-134, mir-138 and miR-124 (Jimenez-Mateos *et al.* 2012; Santarelli *et al.* 2011; Kan *et al.* 2012; Beveridge *et al.* 2010; Peng *et al.* 2013). Since Nova1 is part of miRISC in neurons, it is likely that loss of function mutations in the *Nova1* gene might cause dysregulation of other neuronal miRNAs that are associated with different kinds of neurological disorders. Interestingly, several publications link Nova1 to the pathogenesis of certain neuropsychiatric diseases. For example, NOVA1 was identified in a genome-wide study as promising candidate gene for a genetic predisposition for antipsychotic-induced parkinsonism, a developed side

effect of neuroleptic schizophrenia medication (Alkelai *et al.* 2009). Other studies showed changes in the subcellular distribution of Nova proteins in cortical and hippocampal neurons of mouse brain as well as altered Nova1 mRNA expression in the striatum of rats in response to induced epileptic seizures (Eom *et al.* 2013; Jelen *et al.* 2010). Furthermore, Eom and colleagues propose a Nova-dependent regulation of mRNAs that are associated with epilepsy. Recently, Rusconi and colleagues observed a downregulation of Nova1 in mouse hippocampal neurons in response to pilocarpine-induced status epilepticus (Rusconi *et al.* 2015). A later study uncovered several novel genes, including NOVA1, that are associated with major depressive disorder (MDD) (Malki *et al.* 2013). MDD is a neuropsychiatric disease that has also been linked to miRNA dysregulation in the recent years (Geaghan *et al.* 2015).

In summary, in the presented project a neuron-specific RBP was uncovered in the context of miRNA activity regulation that represents a new modulating factor at the level of miRISC. This study should therefore contribute to a better understanding of the regulatory mechanism that are required for neuronal miRNAs to accomplish their versatile roles in neuronal development and plasticity.

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6 Appendix

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6.2 List of abbreviations

Ago	Argonaute
Apt1	Acyl-protein thioesterase 1
BDNF	brain derived neurotrophic factor
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
co-IP	co-immunoprecipitation
CRE	cAMP response element
CREB	CRE-binding protein
CTX	cortex
Ddx6	DEAD-box helicase 6
Dgcr8	DiGeorge syndrome critical region 8
DHX36	DEAH-box helicase 36
DIV	days in vitro
DNA	deoxyribonucleic acid
Dnd1	Dead end protein homolog 1
<i>E. coli</i>	<i>Escherichia coli</i>
Ewsr1	Ewing sarcoma breakpoint region 1
FCS	fetal calf serum
FMRP	Fragile X mental retardation protein
Fubp1	Far upstream element binding protein 1
FUDR	5-fluorodeoxyuridine
GFP	green fluorescent protein
GTP	guanosine triphosphate
h	hour
HC	hippocampus
HDAC2	Histone deacetylase 2
Hmga2	High mobility group AT-hook 2
HuR	Human antigen R
ICC	immunocytochemistry
Iqgap1	IQ motif containing GTPase activating protein 1
kb	kilobase
KCL	potassium chloride

kDa	kilodalton
LB	Luria-Bertani
Limk1	LIM domain kinase 1
Lin41	Lineage variant 41
Lsm7	Sm-like protein 7
LTD	long-term depression
LTP	long-term potentiation
luc	luciferase
MAP2	Microtubule-associated protein 2
Mef2	Myocyte enhancer factor 2
MEM	minimum essential medium
miRISC	microRNA-induced silencing complex
miRNA	microRNA
MOV10	Moloney leukemia virus 10
mRNA	messenger RNA
mTOR	mechanistic target of rapamycin
mut	mutant
NB	Neurobasal medium
Ncoa3	Nuclear receptor coactivator 3
NMDA	N-Methyl-D-aspartate
Nova1	Neuro-oncological ventral antigen 1
Nxf1	Nuclear RNA export factor 1
Nxt1	Nuclear transport factor 2 like export factor 1
PABP	Poly(A)-binding protein
PCR	polymerase chain reaction
pre-miR	precursor microRNA
pri-miRNA	primary microRNA
PSD-95	Postsynaptic density protein 95
PUM1	Pumilio homolog 1
rAAV	recombinant adeno-associated virus
Rbm25	RNA binding motif protein 25
Rbm4	RNA binding motif protein 4
RLA	relative luciferase activity
RNA	ribonucleic acid

RNAi	RNA interference
RNA-IP	RNA-immunoprecipitation
RNase	ribonuclease
Rpl5	60S ribosomal protein L5
RT-qPCR	Real Time-quantitative PCR
sec	seconds
shRNA	small hairpin RNA
siRNA	small interfering RNA
Smd1	Small nuclear ribonucleoprotein D1
Tnrc6c	Trinucleotide repeat-containing gene 6C
TRBP	Trans-activating response RNA-binding protein
TTX	tetrodotoxin
U2af1	U2 small nuclear RNA auxiliary factor 1
Ube3a1	Ubiquitin-protein ligase E3A 1
V	Volt
WT	wildtype
XRN1	5'-3'-exonuclease 1

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